Universidade de Aveiro Departamento de Biologia
2016

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Respostas multigeracionais em *Daphnia magna*: exposição a pulsos e misturas

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutoramento em Biologia, realizada sob a orientação científica da Doutora Susana Patrícia Mendes Loureiro (Investigadora Auxiliar do CESAM e Departamento de Biologia da Universidade de Aveiro) e do Doutor Amadeu Mortágua Velho da Maia Soares (Professor Catedrático do Departamento de Biologia da Universidade de Aveiro).

Apoio financeiro da FCT e do FSE no âmbito do III Quadro Comunitário de Apoio, através do projeto RePulse-Responses of *Daphnia magna* exposed to chemical pulses and mixtures throughout generations (FCOMP-01-0124-FEDER-019321; Refª. FCT PTDC/AAC-AMB/117178/2010), e pelo projeto "MARPRO-Conservation of marine protected species in Mainland Portugal" através da bolsa de doutoramento atribuída a Ana Rita Rego Gouveia Silva (BD/UI88/5534/2011).

o júri

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agradecimentos

À minha orientadora Dra. Susana Loureiro, por todo o apoio científico, por todas as ideias para melhorar o trabalho, por toda a ajuda e até apoio emocional. Ao professor Amadeu pelas oportunidades.

A todos no laboratório à Patrícia, Diogo, Andreia C., Ana S., Cátia S., Gonçalo, Rui, obrigada a todos pela ajuda no laboratório/discussões científicas. Ao Carlos o companheiro dos (eternos) fins-de-semana no laboratório e discussões de multigerações. Ao Diogo e Maria "grega" por me permitirem fazer *comets* com outros bichos além das dáfnias! À Diana pelas ajudas técnicas e compreensão dos meus stresses. Ao Hugo M. pelas ajudas técnicas e boa música. xD Ao super técnico Abel. Obrigada a todos pelas risadas, pelos ensinamentos nas mais diversas áreas, desde ciência até construção e bricolagem (numa espécie de "Querido mudei o laboratório"). :)

Ao Café pelo amor, paciência e pela felicidade durante todos estes anos juntos. À minha família, pais, irmã, Nuno, sobrinho Afonso por compreender que "a tia não pode porque está a cuidar dos bichos" e à pequenina Inês por ter nascido nesta altura. :) Aos meus amigos de Tondela Filipa, Lucas e Nits. À minha Becas pela "gatoterapia".

Aprendi tanto com vocês!
Obrigada a todos!

palavras-chave

Daphnia magna, multigerações, misturas, carbendazim, triclosan, dano no DNA, pulsos, expressão genética

resumo

O aumento da população humana tem levado a um drástico desenvolvimento nos setores da indústria e agricultura. Consequentemente, quantidades elevadas de químicos, incluindo pesticidas, são diariamente produzidos e utilizados, levando à sua inevitável libertação no ambiente. Uma vez nos ecossistemas aquáticos, os organismos podem estar expostos a químicos por períodos de tempo curtos ou longos. O carbendazim, um fungicida extensivamente utilizado nos campos agrícolas, e o triclosan, um composto antibacteriano usado numa variedade de produtos de higiene pessoal, foram escolhidos para o presente trabalho como químicos representantes de origem antropogénica. Vários estudos demonstraram que ambos os químicos estão presentes nas águas superficiais e são prejudiciais para várias espécies aquáticas. Ademais, num cenário real é mais provável que os organismos estejam expostos a misturas de químicos do que a um químico individualmente. Considerando o anteriormente exposto, um dos objetivos do presente estudo foi determinar os efeitos individuais e em mistura do carbendazim e triclosan utilizando o cladócero *Daphnia magna*, uma espécie modelo amplamente utilizada em ecotoxicologia. Além disso, e considerando que os organismos aquáticos podem estar continuamente expostos a compostos químicos, uma abordagem multigeracional deverá ser considerada. Por este motivo, o presente estudo tem também como objetivo perceber como uma exposição contínua a uma concentração ambientalmente relevante de carbendazim afeta a aptidão da descendência. Para isso, duas experiências multigeracionais foram realizadas, uma até à décima segunda geração (F12) e outra até à décima terceira geração (F13). Adicionalmente, e considerando a libertação dos pesticidas para o ambiente aquático por pulsos, outro objetivo foi perceber como é que a descendência de dáfnias previamente expostas ao carbendazim iria responder a novos pulsos de pesticidas diferentes: a triclosan e à mistura de triclosan e carbendazim. Vários parâmetros individuais, como a sobrevivência, longevidade, imobilização, reprodução e alimentação foram avaliados, assim como alguns parâmetros subcelulares, incluindo genotoxicidade (dano no DNA avaliado pelo ensaio do cometa), diferentes biomarcadores bioquímicos (colinestarase, catalase, glutationa *S*-transferase e peroxidação lipídica), parâmetros energéticos (lípidos, carboidratos, proteínas e energia disponível e consumida) e, por último, alterações na expressão genética (utilizando um microarray personalizado para *D. magna*).

resumo (cont.)

Na exposição individual, o carbendazim mostrou-se mais tóxico do que o triclosan para a *D. magna*, e ambos os compostos causaram dano no DNA. Relativamente à exposição a misturas, diferentes parâmetros seguiram diferentes padrões de resposta, desde aditividade: modelo de ação independente (inibição alimentar e reprodução), até desvios dependentes do nível da concentração da mistura (imobilização) e desvios dependentes da razão entre os componentes da mistura, com sinergismo causado principalmente pelo triclosan (dano no DNA). Os impactos multigeracionais do carbendazim para a *D. magna* foram mais notórios ao nível do dano no DNA (aumentou ao longo das gerações), mudanças na expressão genética e diminuição na longevidade depois de doze gerações. Nas gerações F0 e F12, o carbendazim afetou genes envolvidos na resposta ao stress, replicação e reparação do DNA, neurotransmissão, embriogénese, biossíntese de proteínas, produção de ATP e metabolismo dos lípidos e carboidratos. Considerando os demais parâmetros avaliados, não foi observado um padrão claro de tolerância ao longo das gerações expostas ao carbendazim. No global, as dáfnias em meio limpo e as dáfnias expostas ao carbendazim durante algumas gerações mostraram uma sensibilidade semelhante depois da exposição ao triclosan e também padrões de mistura semelhantes após exposição à mistura binária (carbendazim e triclosan) na experiência por pulsos. O presente estudo realça a importância de usar abordagens multigeracionais (com mais de três gerações) juntamente com múltiplos parâmetros na análise de toxicidade de pesticidas com o objetivo final de melhorar a avalição de risco ambiental.

keywords

Daphnia magna, multigenerations, mixtures, carbendazim, triclosan, DNA damage, pulses, gene expression

abstract

The human population increase led to a drastic evolution in agricultural and industrial sectors. As a result, high quantities of chemicals, including pesticides, are daily produced and used, leading to their inevitable release into the environment. Once in aquatic ecosystems, organisms can be exposed to chemicals for short and/or long-term periods. Carbendazim, a fungicide used extensively in agricultural fields, and triclosan, an antibacterial compound used in a wide number of personal care products, were chosen in the present work as representative of the man-made chemical disposal. Several studies reported that both chemical compounds are present in surface waters and are harmful to several aquatic species. Additionally, in a real scenario it is most likely for organisms to be exposed to chemical mixtures rather than to single compounds. Taking the above statements into consideration, one of the aims of the present study was to determine the effects of single and combined mixtures of carbendazim and triclosan to the cladocera *Daphnia magna*, a test-model species widely used in ecotoxicology. Moreover, bearing in mind that aquatic organisms might be continuously exposed to compounds, a multigenerational approach should be considered. Therefore, the present work also aimed at understanding how a continuous exposure to an environmental relevant concentration of carbendazim affects offsprings´ fitness. For that, two multigenerational experiments were conducted, one testing until the twelfth (F12) generation and other testing until the thirteenth (F13) generation. Additionally, and considering the release of pesticides into the aquatic environment by pulses, another challenge was to understand how offspring from daphnids previously exposed to carbendazim respond to new pesticide inputs in two different situations: triclosan alone and the mixture of carbendazim and triclosan. Several individual endpoints, such as survival, longevity, immobilisation, reproduction and feeding activity, were evaluated and also several subcellular endpoints, including genotoxicity (DNA damage evaluated through the comet assay), several biochemical biomarkers (cholinesterase, catalase, glutathione *S*-transferase and lipid peroxidation), energy-related parameters (lipids, carbohydrates, proteins and available and consumed energy) and finally changes in gene expression (using a *D. magna* custom microarray).

abstract (cont.)

In the single exposure, carbendazim was more toxic to *D. magna* than triclosan, and both compounds caused DNA damage. Concerning mixture exposures, different endpoints followed different patterns of response, from additivity: Independent Action model (feeding inhibition and reproduction), to deviations for dose level dependency (immobilisation) and dose ratio dependency, with synergism mainly caused by triclosan (DNA damage). Multigenerational impacts of carbendazim to *D. magna* were mainly observed in terms of DNA damage (increased throughout generations), changes in gene expression and a decrease in longevity after twelve generations. In both F0 and F12 generation, carbendazim affected genes involved in response to stress, DNA replication/repair, neurotransmission, embryogenesis, protein biosynthesis, ATP production, lipids and carbohydrates metabolism. No clear pattern towards tolerance was found throughout the generations exposed to carbendazim regarding the other ecotoxicological endpoints. Overall, daphnids in clean medium and daphnids exposed to carbendazim for some generations showed similar sensitivity after exposure to triclosan and also similar mixture patterns after the binary mixture (carbendazim and triclosan) exposure in the pulse experiment. This research highlights the importance of using multigenerational approaches (with more than three generations) along with multiple endpoints to understand pesticide toxicity with the ultimate goal of improving the environmental risk assessment.

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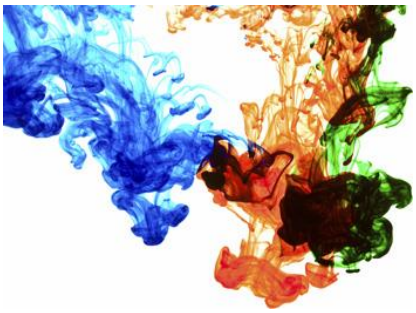
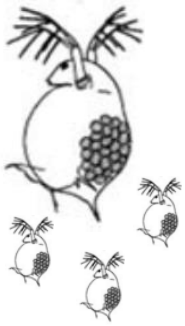
Chapter 1

General Introduction





1. General introduction

1.1 Contamination of aquatic environments

Climate and anthropogenic factors have been raising environmental concerns, including loss of water availability due to contamination of surface and groundwater and loss of habitat of numerous species of flora and fauna all over the years (Ecobichon, 2001; Hansen *et al.*, 2001).

The increase in human population has created a great demand for freshwater, among other ecosystems. However, this increase in population also leads to an increasing application of several chemical compounds in agriculture, industry, medical treatment and household products and, consequently, to an increase on its disposal. Several examples of widely used chemicals from different sources can include pharmaceuticals, hormones, organic compounds (Kolpin *et al.*, 2002; Ortiz de Garcia *et al.*, 2013), metals (Elkady *et al.*, 2015; Nascimento *et al.*, 2015) and nanoparticles (Farkas *et al.*, 2011; Liu *et al.*, 2013). Intense application of agrochemicals culminates in aquatic contamination with pesticides as well.

Pesticides are a highly diverse group of anthropogenic compounds which have several applications, including in urban/industrial activities and agricultural areas. Considering the release of such diverse number of different chemicals into the environment, contamination is frequently composed by a combination of various stressors (Gordon *et al.*, 2012). These combinations of chemicals might be a consequence of different sources of pollution present in the environment. Some examples include: the release of untreated wastes to domestic sewage systems or directly to surface waters; metabolic excretion of compounds and disposal into sewage systems; overflow of untreated sewage due to system failures or extreme rain events; use of biosolids (sewage solids) for fertilization of agricultural soils; release of pesticides from agricultural crops (spray drift), among others (Fig. 1.1).

The fate of chemicals in the environment is influenced by their own properties, where persistence (half-life), mobility and biodegradability play a major role (Gavrilescu, 2005). During spray application, pesticides are emitted through the air, contaminating the air and soil, not only in the area where they are applied but also in distant areas (Felsot *et al.*, 2011). Some of the chemicals tend to remain in soils and sediments and others can be

leached to surface and/or groundwater (Gavrilescu, 2005). Furtheremore, air, food (*e.g.* agricultural products) and organisms' tissues might be contaminated with pesticides as well (Gavrilescu, 2005).

Figure 1.1 Conceptual diagram with possible origins and fate of different chemical compounds. Adapted from Christian G. Daughton, U.S.EPA-Las Vegas (original February 2011).

As regards to organisms effects, the toxicity depends on the available and accessible fractions of the chemical (bioavailability and bioaccessibility), which varies depending on the chemical (*e.g.* chemical structure), environmental conditions, organisms physiology and life-traits, among other factors (Phyu *et al.*, 2004). Hydrophobicity/lipophilicity is an important physicochemical property which determines the affinity of a molecule for lipophilic environments. Knowledge on the lipophilicity might indicate if the substance has the potential to bioaccumulate, usually where a log K_{ow}>5 indicates a high potential of bioaccumulation (Coogan *et al.*, 2007). Additionally, lipophilic chemicals usually have a longer half-life. The octanol/water partition coefficient (K_{ow}) is an indicator of lipophilicity, and varies depending on the media pH. Jointly with pH, temperature, organic matter content and hardness are other physicochemical factors influencing the impact of chemicals in the aquatic environment (Rand and Petrocelli, 1985).

Nowadays, the increasingly use of chemical compounds leads to the contamination of aquatic ecosystems with possible negative consequences to several organisms. Pesticides disturb organisms from different trophic levels in aquatic ecosystems (DeLorenzo *et al.*, 2001), including phytoplankton (*Pseudokirchneriella subcapitatum*), zooplankton (*Daphnia magna*) (DeLorenzo *et al.*, 2002) and fish (*Oncorhynchus mykiss)* (Capkin *et al.*, 2006). Consequently, organisms from higher trophic levels and thus human health might be affected as well.

In the present study two compounds were selected: carbendazim and triclosan. Carbendazim (CBZ, methyl-2-benzimidazole carbamate) has been used throughout the years as a fungicide in plant production products' and it may pass into aquatic systems through run-off, drainage (due to, for instance, rainfall events) or direct spray-drift to crops and soil. Carbendazim is used to inhibit fungi in several fruit crops, cotton, sugarcane, tobacco, peanuts, vegetables and cereals (WHO, 1993). Carbendazim was found at concentrations of 4.5 µg/L in surface waters (Palma *et al.*, 2004) and it was listed as a priority substance by the European Commission due to its endocrine disruptor effects (European Commission, 1999). Carbendazim was detected in fresh fruits, including in apples (Lozowicka, 2015). Triclosan (TCS, 5-Chloro-2-(2,4-dichlorophenoxy)phenol) is a biocide used in everyday life products, such as personal care products and it is approved by EU Cosmetics Directive (Council Directive, 1999 edition). Toothpastes, mouth rinses, hand disinfecting soaps and deodorants are examples of personal care products containing triclosan (Gaffar *et al.*, 1994). In addition, it is present in other frequently used products, such as plastics, detergents, household sponges, shoes, textiles (*e.g.* socks, underwear, bed clothes, carpets), food packaging materials, furniture and toys (EPA, 2003a, 2008; Jones *et al.*, 2000). Triclosan was found in aquatic sediments and in biosolids from wastewater treatment plants (WWTP) that are intended for land application (Chalew and Halden, 2009). Due to run-off, triclosan might end in surface waters. In addition, triclosan is a widely used compound in consumer products, which is washed down the drain and ends up in domestic wastewater in municipal WWTP, where the removal rates are not 100% (Ying and Kookana, 2007). Triclosan has been also associated with bacterial resistance and consequently antibiotic resistance (Chuanchuen *et al.*, 2001). Contamination with triclosan

is not only restricted to aquatic/terrestrial environment, triclosan was detected in human milk samples (Adolfsson-Erici et al 2002).

1.2 Time of exposure in ecotoxicology

The presence of pesticides in the environment has been extensively reported. Although pesticides are designed to act upon a given organism (insects, bacteria, fungi, *etc*.), through a specific mode of action, *i.e.* in a specific target (site), their presence in the environment may pose a threat to non-target organisms.

Chemical analyses provide important information regarding the presence and concentration of chemical compound(s) in the environment. However, chemical analyses do not provide a precise prediction of harmful effects to organisms, since the effects depend on, for instance, the bioavailibility of the chemical (Phyu *et al.*, 2004). Thus, ecotoxicological tests are considered crucial tools for hazard assessment.

To determine chemical hazard, standard ecotoxicological tests, for instance immobilisation (acute) and reproduction tests with *Daphnia* or life-cycle tests with chironomids, have been used (OECD, 2004, 2008, 2010). However, since long-term tests are more expensive and time-consuming, short-term tests, *e.g.* acute tests, are sometimes preferred. Though and bearing in mind that in the environment organisms might be continuously exposed to a given chemical, it is essential to study the effects at long-term, for instance using multigenerational experiments. These experiments can provide useful information to bridge the knowledge from the individual to the population level and improve environmental risk assessment (REACH, 2006), since maternal exposure might have consequences on the offspring, leading to a higher or less sensitive offspring (*e.g.* through adaptation) and to the subsequent generations (Brausch and Salice, 2011; LaMontagne and McCauley, 2001). Therefore, long-term exposures are important for decision-making in risk assessment with the ultimate purpose of protecting the environment.

Within different times of exposure, accounting for effects at different levels of organization is of utmost importance. The study of effects from the individual to higher organizational levels, population level, might be crucial to anticipate potential ecological effects in short and long-term exposures as well.

1.3 Mixture toxicity and pulse exposure

Considering all the above mentioned, in natural ecosystems organisms are frequently exposed to a cocktail of chemicals. Recognizing this, an important challenge is to estimate the interactive effects of chemicals to improve the environmental risk assessment. In addition, the Framework for Cumulative Risk Assessment highlights the need to analyse the combined risks to human health and multiple stressors in the environment (EPA, 2003b).

The mode of action (MoA) of a chemical is defined as a set of biochemical, physiological and/or behavioural signs resulting from the exposure of an organism to a stressor (*e.g.* chemicals) (McCarty, 2002), and it is an important point to consider in mixture testing. The notion of MoA is different in human toxicology and ecotoxicology, being in the last one broader and with different toxic effects for the biological community (bacteria, plants and animals) (EFSA, 2015). Additionally, a single chemical might have multiple MoA and the complexity in identifying the MoA is a challenge in predicting mixture effects (EFSA, 2015). This could provide more information to fill data gaps in toxicological/ecological hazard assessment, including for instance the Adverse Outcomes Pathways (AOP) (EFSA, 2015). AOP methodology describes the development of events from the molecular level to the individual and population level (OECD, 2013).

In the current risk assessment, the evaluation of combined effects of chemicals usually can be predicted using two conceptual models, the Independent Action (IA) and Concentration Addition (CA) models. Both models are based on non-interaction between chemicals/stressors, assuming that each component does not interact/influence the biological action of the other component within a mixture (Hewlett and Plackett, 1959). The IA model is used for dissimilarly acting substances (different MoA) and the mathematical formulation is based on probability of responses and is expressed as follows:

(Equation 1) $Y = \mu max \prod_{i=1}^{n} qi\ (Ci)$

Where Y denotes the biological response, C_i is the concentration of chemical i in the mixture, $q_i\ (C_i)$ the probability of non-response, μ_{max} the control response for endpoints and $\prod$ the multiplication function.

On the other hand, the CA model is generally used for similarly acting substances (similar MoA), where components are assumed to act as dilutions of the others and mathematically it can be expressed as:

(Equation 2) $\sum_{i=1}^{n} \frac{Ci}{ECxi} = 1$

Where *ci* denotes the concentration for chemical *i* in the mixture and EC_{xi} is the effect concentration of chemical *i* that results in the same effect as the mixture. This quotient c_i/EC_{xi} is commonly referred as Toxic Unit (TU) value as well, representing the contribution to toxicity of the (individual) chemical *i* in a mixture.

Since chemicals might interact inside the organisms, deviations from both reference models can occur such as synergism or antagonism, dose ratio or dose level dependency (Jonker *et al.*, 2005; Loureiro *et al.*, 2010). These deviations can be evaluated using the MIXTOX tool proposed by Jonker and colleagues (Jonker *et al.*, 2005). The first deviation corresponds to synergism or antagonism, being the synergistic effect characterized by a greater than additive effect (more severe effect) and the antagonistic effect characterized by a less than additive effect (less severe effect) (Bliss, 1939). The following deviations are the dose level deviation (DL), where the toxicity depends on the dose and differ at low or high doses of the chemicals; and the dose ratio dependent deviation (DR), where the toxicity depends on the mixture composition and the toxicity is mainly caused by one of the chemicals (Jonker *et al.*, 2005).

Throughout the years, several works proved that chemical mixtures do not always follow the classic models of mixture toxicity. Compounds interacted and reflected patterns of antagonism or synergism when in mixture, including in studies using *D. magna* (Loureiro *et al.*, 2012; Pavlaki *et al.*, 2011).

In the present study, the IA model was used as a starting point to determine the response pattern of the binary mixture of carbendazim and triclosan, since the exactly MoA of the two selected compounds is not completely known on the tested organism (the water flea *D. magna*). However, recently the EFSA report from the Scientific Colloquium 21 on the "Harmonisation of Human and ecological risk assessment of combined exposure to multiple chemicals" recommended the use of the CA model even for dissimilarly acting chemicals (EFSA, 2015). Thus, data modelled by the CA model will be presented in

supplementary material. More details will be presented on the next chapters. The EFSA report states that distinctions regarding MoA used to decide which reference model to choose is sometimes hard and in the literature the IA model did not provide a more conservative prediction comparing with the CA model (Backhaus *et al.*, 2004; EFSA, 2015). In addition, the need to develop methods to evaluate mixture effects with chemicals with independent/dissimilar MoA and alternative methods to animal testing were discussed in the same report (EFSA, 2015).

In addition to constant exposure or to mixture exposure, episodic exposure to chemicals occur, for instance, after accidental release, sewage treatment plants overflow, after pesticides application and/or runoff from fields due to rain events. This might result in short-term pulse exposures, with chemicals entering in the environment at different times (Liess *et al.*, 1999). Consequently, aquatic organisms that live in adjacent areas might experience episodic exposures for different periods of time. These chemical pulses are dynamic and might ensue singly or in mixture and last for hours to days (Gordon *et al.*, 2012). Additionally, the toxic effects of chemical pulses depends on the concentration of the chemical (high or low concentration), generally with low concentrations presenting a possible recovery after the stressor exposure (Gordon *et al.*, 2012). The European Commission's Technical Guidance Document on Risk Assessment of Chemicals addresses the issue of intermittent releases (European Commission, 2003), demonstrating that throughout the years this issue is gaining importance. Therefore, adding extra stressors, such as chemical pulses, to hazard assessment processes may straighten the knowledge gap that exists in risk assessment. This issue was addressed in one of the chapters of the present thesis and will be discussed in more detail later.

1.4 Multigenerations: from subcellular to individual level

Multigenerational studies are of extreme relevance in order to evaluate the impact of a continuous exposure to one chemical (Pane *et al.*, 2004). In fact, organisms of previously exposed mothers might have an increase in tolerance, becoming less sensitive to the compound or, on the opposite side, a magnified (toxic) effect might also occur. In multigenerational tests several species have been used, from aquatic organisms including the amphipod *Hyalella azteca* (Borgmann *et al.*, 2007), the crustacean *Artemia*

(Sukumaran and Grant, 2013), the zebrafish *Dania rerio* (Coimbra *et al.*, 2015), the aquatic insect *Chironomus riparius* (Vogt *et al.*, 2007), to soil organisms including the earthworm *Eisenia fetida* (Schnug *et al.*, 2013) and the springtail *Folsomia candida* (Paumen *et al.*, 2008).

The focus of the present work will stand on the freshwater cladoceran *D. magna* (Fig. 1.2), which will be used as model organism for multigenerational testing. The microcrustaceans of the genus *Daphnia* are important zooplanktonic herbivores in freshwater, as they are widely geographic distributed and occupy a central position in food webs, linking primary producers with consumers (Lampert, 2006; Lampert and Sommer, 2007). *D. magna* has demonstrated to be a sensitive organism to several compounds, including to pesticides (Ferrando *et al.*, 1992; Toumi *et al.*, 2015). *D. magna* reproduces asexually by parthenogenesis, eliminating the genetic variability between organisms and making daphnids suitable organisms for multigenerational experiments (Baird and Barata, 1998). On the other side, environmental stresses might trigger sexual reproduction on *Daphnia* sp. (Paland et al. 2005).

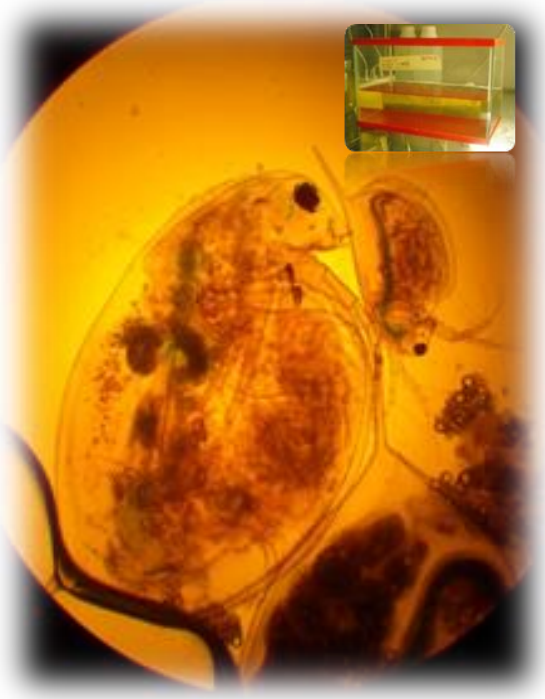


Figure 1.2. *Daphnia magna* adult and neonate and culture aquarium (top corner), Source: Rita Silva.

Several multigenerational tests using *D. magna* have been conducted with several different compounds, from pesticides (Sanchez *et al.*, 2004) and metals (Bodar *et al.*, 1990; Guan and Wang, 2006) to antibiotics (Kim *et al.*, 2014) and nanomaterials (Arndt *et al.*, 2014). However and to our knowledge, none of them integrates a battery of tests from subcellular to population effects as in the present thesis. Subcellular endpoints (*e.g.* DNA damage, biochemical biomarkers or energy-related parameters, gene expression, *etc.*) might provide valuable new information about the influence of compounds. In addition, the toxic effects at the subcellular level might sometimes help to anticipate individual effects and might help to understand changes in sensitivity of organisms throughout generations.

Carbendazim was chosen as a model-chemical in this multigenerational approach due to some of the already known characteristics/effects. It was chosen mainly because it affected the reproduction capacity of *D. magna*, reducing the number of neonates and causing aborted eggs (probably related with mitosis inhibition) in considerably low concentrations (Ribeiro *et al.*, 2011; Chapter 2 - Silva *et al.*, 2015). In addition, this compound caused immobilisation, feeding activity reduction and DNA damage in this organism (Ribeiro *et al.*, 2011; Chapter 2 - Silva *et al.*, 2015). Despite carbendazim has been prohibited in some countries, it is still applied in others and in a variety of different crops (from fruits, vegetables, seeds to cereals, *etc.*) (EU Pesticide Database, 2015), contributing to a continuous release that might last for two or more seasons. Several subcellular endpoints have been studied in the present thesis, including for instance genotoxicity, which was evaluated using the comet assay (detects DNA strand breaks in single cells) (Singh *et al.*, 1988). DNA damage is considered an important endpoint since it might influence not only survival, but also growth and reproduction (De Coen and Janssen, 2003), for that reason, efforts should be made to test whether a compound exerts genotoxicity. Additionally, genotoxic compounds have the capacity to alter DNA replication, with possible consequences on genetic transmission (Combes, 1992).

The biomarkers cholinesterases (ChE), catalase (CAT), glutathione *S*-transferase (GST) and lipid peroxidation (LPO) were also evaluated in the present study. These biomarkers of effect can provide a fast biological response in individuals exposed to different chemicals, being considered measures of the initial changes caused by chemicals.

The interaction between the compound and the biological receptor site induces effects that start at the subcellular level and might lead to adverse effects at higher levels of organization. Relationships between effects at different levels of biological organization are relevant, even though they are sometimes difficult to make (De Coen and Janssen, 2003).

In the aquatic environment, when organisms are exposed to contaminated media, this might imply a trade-off in energy allocation from, for instance, reproduction and detoxification mechanisms (Calow and Sibly, 1990). For that reason, energy reserves including lipids, carbohydrates, proteins and available and consumed energy were evaluated as well.

Additionally, when organisms are exposed to some chemicals, and consequently are in stress, their epigenome might be altered (changes in epigenetic marks).

Epigenetics describes changes in gene expression in organisms and these changes can alter organism's phenotype (Harris *et al.* 2012). The concept of epigenetics has evolved throughout the years, one of the last definitions is "Epigenetics is the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence" (Vandegehuchte and Janssen, 2011). Several mechanisms are involved in epigenetics, including DNA methylation, histone tail modifications and non-coding RNA-associated gene silencing (Vandegehuchte and Janssen, 2011). These epigenetic changes might be transferred from mothers to offspring and might possibly have consequences to subsequent generations. *Daphnia* was the first crustacean to have its genome sequenced (Colbourne *et al.*, 2011). With the genome sequence, microarrays can be developed to better understand changes in the expression of thousands of genes in one organism, and consequently comprehend molecular mechanisms of toxicity (Harris *et al.*, 2012; Soetaert *et al.*, 2007). Using a custom *D. magna* cDNA microarray, gene expression profiles will be evaluated in the present work in the multigenerational test. The *Daphnia* microarray might indirectly shed some lights regarding this issue, on changes in gene expression throughout the generations, and looking at the possibility of a chemical acting on the daphnids epigenome.

1.5 Aims of the study and Conceptual framework

Despite of what is known about the negative effects of pesticides in the environment, pesticides are still needed and are widely applied (Ecobichon, 2001). Several stressors, including pesticides, are responsible for environmental changes and therefore there is a continuous need to know how species will respond to these changes. Therefore, the general aim of the present study was to evaluate and understand the effects caused by a long-term (multigenerational) exposure to a model chemical in *D. magna*.

With that purpose, several specific goals were established. One of those was to estimate the effects of single and combined chemicals (mixture exposure) to *D. magna*. Carbendazim and triclosan were chosen considering all the above mentioned and the effects were evaluated using standard ecotoxicity test, complemented with an additional test, the comet assay.

The present study also sought to understand how a continuous exposure to a model chemical, carbendazim, affected organism's fitness. For that, the cladocera *D. magna* was exposed to an environmental relevant concentration of carbendazim throughout twelve generations and several endpoints were evaluated. The multigenerational approach will provide clues about the sensitivity shifts to stressors among different generations, if and how organisms recover after different periods of exposure and whether future generations will acclimate or adapt at some level to these contaminated scenarios. In addition, a shift or step from the individual to the population level was also aimed to be achieved.

Considering that conditions in the aquatic environment are not static and that pulses of chemicals might enter not simultaneously, another challenge was to understand how daphnids from generations previously exposed to a given chemical will respond to new inputs of different chemicals.

The standard ecotoxicity tests, immobilisation and reproduction tests, were carried out and the feeding inhibition test was used as a trait-based approach. Additionally, the comet assay (DNA damage), the biomarkers (ChE, CAT, GST and LPO), the energy reserves (lipids, carbohydrates, proteins and available and consumed energy) and the *Daphnia* microarrays were used in order to provide information regarding the gene expression changes caused by carbendazim.

Throughout the work the following questions were raised:

1) Do offspring whose progenitors were exposed to contaminated scenarios respond the same way as their progenitors?
2) Will an exposure to a stressor modify the response of future generations to mixture exposure when the previous (single) exposure was present?
3) Do pulse exposures induce similar results as simultaneous ones?
4) Can daphnids recover from stressor exposure?
5) Will changes in exposures induce sensitivity turnovers within organisms' generations?

To answer these questions, this thesis was divided in several studies that are reflected in five chapters of this thesis (chapters 2 to 6).

Chapter 1 corresponds to the General Introduction.

Chapter 2 describes the effects of triclosan and carbendazim in single exposures, and their binary mixture using *D. magna*. For that purpose, acute immobilisation, reproduction and feeding inhibition tests were carried out and the comet assay was also used to unravel genotoxic effects (DNA damage).

Chapter 3 describes the long-term effect of carbendazim in *D. magna,* focusing on the effects to the offspring, upon parental exposure. For that, *D. magna* was exposed for twelve successive generations to carbendazim and effects on immobilisation, feeding activity, reproduction and *in vivo* genotoxicity were assessed on neonates throughout generations.

Chapter 4 investigated how *D. magna* exposed throughout generations to carbendazim reacted upon pulse exposures to other chemicals (in this case triclosan) and the mixture of both chemicals (the pre-exposed and the new pulse). Within this, *D. magna* were continuously exposed during twelve generations to carbendazim and exposed to pulses of triclosan and of triclosan and carbendazim (as a mixture) at some of the generations. The induced effects of pulses on daphnids previously exposed to carbendazim and kept in clean medium were compared regarding mobility (immobilisation test) and DNA integrity (comet assay).

Chapter 5 describes the long-term effects of carbendazim (in a thirteen generation experiment) on survival/longevity, reproduction, length of mothers, DNA damage (comet

assay), biomarkers (ChE, CAT. GST and LPO) and energy reserves (lipids, carbohydrates, proteins and available and consumed energy), assessed at some generations of *D. magna*.

Chapter 6 describes the long-term effects of carbendazim at the gene transcription level in a multigenerational test (F0-F12) using a *D. magna* custom microarray. As one of the specific aims was to better understand molecular responses of carbendazim on *D. magna*, two questions were emphasized in this chapter: *i)* what changes are induced by carbendazim in the *D. magna* transcriptome and *ii)* if this changes are kept in time with continuous exposure.

Finally, Chapter 7 corresponds to a General Discussion of the main results and Conclusions.

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Chapter 2

Ecotoxicity and genotoxicity of a binary combination of triclosan and carbendazim to Daphnia magna

Published by Ecotoxicology and Environmental Safety 115 (2015) 279–290

doi:10.1016/j.ecoenv.2015.02.022

Ecotoxicity and genotoxicity of a binary combination of triclosan and carbendazim to *Daphnia magna*

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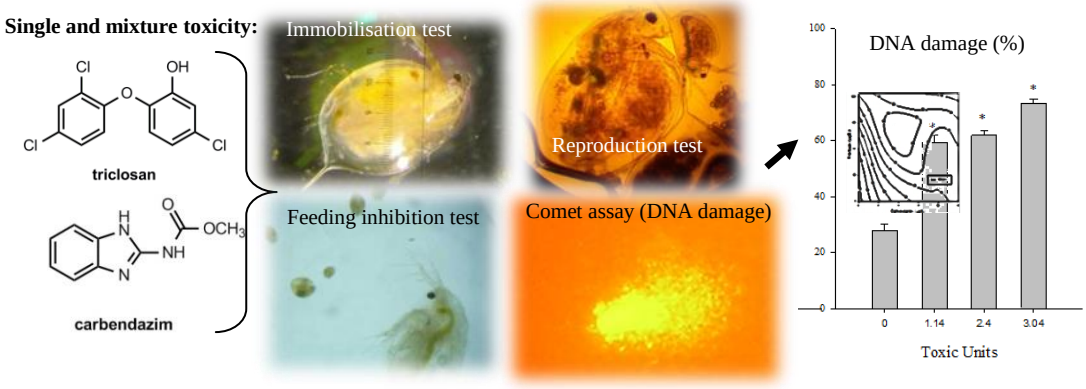

Abstract

In the environment, chemical substances appear as complex mixtures and consequently organisms are exposed to a variety of chemicals from different sources (*e.g.* wastewater treatment plants, agriculture runoffs). When studying chemical mixtures, there are two conceptual models usually used to predict toxicity: the Independent Action (IA) and Concentration Addition (CA) models. However, deviations from these reference models can occur as synergism or antagonism, dose ratio or dose level dependency. The aim of the present study was to investigate the effects of triclosan and carbendazim, and their binary mixture to *Daphnia magna*. With this purpose, immobilisation, feeding inhibition, and reproduction were assessed as main ecotoxicity endpoints. In addition, i*n vivo* genotoxicity of both chemicals was investigated using the comet assay. In the single exposure, carbendazim was more toxic to *D. magna* than triclosan. When daphnids were exposed to both single compounds, DNA damage was observed. Concerning mixture exposures, different endpoints followed different patterns of response, from additivity: IA model (feeding inhibition and reproduction data), to deviations that indicate interaction between chemicals inside the organism: dose level dependency (immobilisation data) and dose ratio dependency (DNA damage). This study showed that additivity does not rule the dose-effect relation in chemical mixtures of carbendazim and triclosan and interactions between both chemicals might induce generally higher toxicity than predicted based on single chemical exposures.

Key words: single/mixture toxicity; DNA damage; reproduction; feeding inhibition; synergism/antagonism

1. Introduction

During the last decades, the use of pesticides continued to increase, being one of the major sources of pollution for aquatic ecosystems. In addition, other frequent sources of pollution occur from Wastewater Treatment Plants (WWTP) effluents, contributing therefore to the presence of complex mixtures in aquatic systems. The risk assessment of these complex mixtures can be underestimated because procedures account for single

toxicity of each chemical and usually do not account for their additivity or possible interactions (Loureiro *et al.*, 2010; Pavlaki *et al.*, 2011).

Given the increasing importance of studying mixtures, this study will provide information regarding the toxic effects of two widely used compounds with possible different origins: carbendazim is applied directly in agricultural crops whereas triclosan is used in many consumer products ending up in wastewater treatment effluents. Usually studies on mixture effects are performed using co-occurring compounds from the same origin, and few focus on the interactions of chemicals from different sources. Nevertheless, both compounds enter in the aquatic environment (to surface and groundwater) and can be used as model compounds to understand mixture effects (Lishman *et al.*, 2006; WHO, 1993). Triclosan (TCS) is an antimicrobial agent used in several personal care products, including toothpastes, mouthrinses (Council Directive, 1999 edition; Gaffar *et al.*, 1994) and in other frequently used products, such as plastics, shoes, textiles and food packaging materials (Jones *et al.*, 2000). Triclosan has been used in almost every part of the World including in the European Union, where approximately 350 tons of triclosan are produced annually (Singer *et al.*, 2002). Triclosan has been detected in wastewater treatment effluents at concentrations between 0.01 and 2.7 µg/L (Lishman *et al.*, 2006; Reiss *et al.*, 2002), mainly due to the fact that after entering WWTP, it is not completely removed and consequently it is released to the environment (Bester, 2003). In Portugal, triclosan was detected in urban wastewater samples at low concentrations: 124.1 ng/L (Neng and Nogueira, 2012). Other concern is that even after triclosan prohibition in some countries, it stills remains a problem as it aggregates in wastewater sludge and it might be transferred to water environments, persisting for months to years (Lygina *et al.*, 2013). Carbendazim (CBZ) is an active ingredient in systemic fungicides and therefore is largely used in agriculture in several cultures of cereals, sugar, *etc.* (WHO, 1993). Likewise, carbendazim is still autorized at a national level in some European countries including in the United Kingdom and Portugal (EU Pesticide Database, 2015). Carbendazim has been detected at concentrations of 4.5 µg/L in aquatic systems, whose origin is mainly from agricultural runoffs (Chatupote and Panapitukkul, 2005; Palma *et al.*, 2004). Despite the benefits of their application, these compounds are known to be toxic to several aquatic organisms, including the water flea *Daphnia magna* (Daam and Brink, 2007; Ferreira *et al.*, 2008; Orvos *et al.*, 2002; Raut and Angus, 2010; Slijkerman *et al.*, 2004).

Currently, standard ecotoxicology tests mainly focus on the endpoints of mortality and reproduction, providing an effect prediction at the individual and population levels (Hutchinson *et al.*, 2006). In addition, other endpoints closely related to organisms' ecological functions or chemicals' modes of action can also provide crucial information on chemical toxicity and their potential interaction when in mixtures. Feeding inhibition is one of the endpoints that has been used to assess mixture toxicity effects in *Daphnia magna* (Ferreira *et al.*, 2008; Loureiro *et al.*, 2012; Loureiro *et al.*, 2010), which will provide information on the organisms' function as a filter feeder. Regarding endpoints devoted to chemicals' mode of action, the comet assay is a simple and well-established method that measures the DNA damage in single cells. These DNA strand breaks are sensitive biomarkers of genotoxicity (Collins *et al.*, 1997; Singh *et al.*, 1988). The importance of the comet assay relies on the fact that some effects may not be detected using general endpoints (*e.g.* growth or reproduction) and at lower concentrations DNA damage may occur and therefore can be measured as an early warning tool (Jha, 2008). Furthermore, *Daphnia* reproduces asexually, by parthenogenesis, meaning that there is no recombination, making these organisms more vulnerable to DNA damage across generations (Hebert and Ward, 1972; Simon *et al.*, 2003; Sukumaran and Grant, 2013).

Under realistic conditions, organisms are exposed to different and multiple stressors, discharged into the environment from different sources. Usually mixture toxicity prognostic evaluation is based on combining chemicals from similar source of origin. Therefore, mixtures composed of chemicals from different origins that can co-occur in the environment are usually disregarded. To accurately evaluate their effects, modes of action (MoA) should be considered specifically to the study organism. Carbendazim acts on cell division, inhibiting the development of the germ tubes in the nucleus and inhibiting the reproduction capacity in *D. magna* (Canton, 1976). Also carbendazim showed to increase the number of aborted eggs, and a hypothesis was raised on the mitosis inhibition during the eggs division in the brood pouch (Ribeiro *et al.*, 2011). The biocide triclosan inhibits the enoyl-acyl carrier protein reductase (ENR), which is involved in the bacterial lipid biosynthesis, so bacteria are unable to reproduce (Heath and Rock, 2000). In *D. magna*, triclosan enhances the activity of glutathione *S*-transferase (GST), and decreases the superoxide dismutase (SOD) activity, which may indicate damage in the cell membranes, confirming that triclosan causes oxidative stress (Peng *et al.*, 2013).

To predict mixture toxicity effects, there are two conceptual models based on non-interaction between chemicals: the Independent Action (IA) and Concentration Addition (CA) models. The IA model assumes that chemicals have different MoA and the CA model assumes that chemicals have the same MoA, being both generally additive models. Considering that the specific MoA of these compounds in daphnids is still vague, but it is assumed to be different, the IA model was used to predict mixture toxicity in the present study. When additivity is not achieved as the main output result due to interactions that may occur between chemicals, deviations from the two conceptual models (IA and CA) can be observed, such as synergism (more severe effect), antagonism (less severe effect), or dose level dependent deviation (DL: deviations differ at low or high doses of the chemical) and dose ratio dependent deviation (DR: deviations depend on the composition of the mixture). To evaluate these deviations, the MIXTOX is an available tool to be used (developed based on Jonker *et al.* (2005)). The toxic unit (TU) approach quantifies the relative contribution of each chemical to the toxicity in a mixture and can be calculated as follows:

(Equation 1) $TU=c/EC_{x.}$

Here, c represents the actual concentration of a chemical required to produce a certain effect, EC_x (effect concentration) (Bliss, 1939; Jonker et al., 2005; Loewe and Muischnek, 1926; Loureiro *et al.*, 2010).

To our knowledge only few studies have investigated the toxic effects of triclosan in *D. magna* (Flaherty and Dodson, 2005; Orvos *et al.*, 2002) and none addressed the effects of the binary mixture of triclosan and carbendazim. Therefore, and due to their potential co-occurrence from different sources, studies evaluating and predicting their joint effects are helpful to derive more accurate risk assessment results when both compounds are found in the environment.

For all the above mentioned, the aim of the present study was to investigate the effects of triclosan and carbendazim single exposures, and their binary chemical mixture to *D. magna*. Acute immobilisation and reproduction tests were carried out as standard ecotoxicity tests, the feeding inhibition test was used as a trait-based approach, and the comet assay to unravel genotoxic effects. Interactions between compounds are important to regulatory authorities in Europe (and US), and they are imperative when resulting into synergistic patterns (several cases of synergism are reported in literature in the presence of

pesticide mixtures), and therefore this study will highlight additionally the prediction of toxicity patterns that may occur when these two compounds are present in the environment (Cedergreen, 2014).

2. Materials and Methods

2.1 Test organism

D. magna Straus clone K6 (originally from Antwerp, Belgium) was obtained from continuous culture maintained in a laboratory at the University of Aveiro (Portugal) and cultured in American Society for Testing and Materials (ASTM) moderated-hard-water medium (ASTM, 1980), with a temperature between 19^0C and 21^0C and a 16h light-8h dark photoperiod. *D. magna* cultures consisted of 6 L glass aquariums containing 3 L of culture medium and 50 daphnids. The medium was renewed three times a week and daphnids were fed with *Raphidocelis subcapitata* (formerly known as *Pseudokirchneriella subcapitata*) at a concentration of $3x10^5$ cells/mL and supplemented with an organic extract (Marinure seaweed extract, supplied by Glenside Organics Ltd.) (Baird *et al.*, 1989). All tests were performed with *D. magna* neonates from the third to fifth broods.

2.2 Test chemicals

Stock solution for triclosan (Irgasan, CAS No. 3380-34-5, 97% purity, Sigma-Aldrich) and carbendazim (CAS No. 10605-21-7, 99.4% purity, Bayer) were prepared in ASTM with acetone and then used for preparing the exposure treatments in ASTM medium. Therefore, a solvent control of 100 µL acetone/L was also included in all experimental setups as the maximum concentration recommended in the OECD guideline 23 (OECD, 2000).

Chemical analyses were performed to measure concentrations of triclosan and carbendazim in the test medium at Marchwood Scientific Services, Southampton, UK. The analyses for triclosan were performed by Gas Chromatography-Mass Spectrometry (GCMS-MS). A representative portion of the sample (200-300 mL) was extracted with 20 mL of acetonitrile (containing 1% acetic acid). The sample was then subjected to a solid phase extraction stage using a 200 mg cartridge. A methanol wash followed and 10µl final

injection volume applied. Standards were prepared in solvents at seven levels with recoveries in the range 70-120%. The analyses for carbendazim were performed by Liquid Chromatography-Mass Spectrometry (LCMS-MS) using the Querchers method. A representative portion of the sample (200-300 mL) was extracted with 20 mL of acetonitrile (containing 1% acetic acid). This was followed by a partitioning step with magnesium sulphate and a subsequent buffering step with sodium acetate. After mixing an aliquot with methanol, the extract was injected directly into the LCMS-MS system (instrument Agilent 6410 Triple Quad LCMs-MS) without any clean-up. A 10 µl injection volume was utilized. Standards were prepared in solvents at seven levels with recoveries in the range 70-120%. The model used assumed that concentrations of the chemical decreased with time, and the change in concentration was given by the following equation:

(Equation 2) $C_t = C_0 e^{-k0t}$

Where C_0 corresponds to the initial external concentration (µg/L), K_0 corresponds to the constant of degradation of the chemical in the medium (/hour) and t corresponds to time (hours) (Widianarko and Van Straalen, 1996).

2.3 Single chemical testing

2.3.1 Immobilisation test

Acute tests were performed according to the OECD 202 guideline (OECD, 2004). Daphnids with less than 24 hours were used to initiate the test. After 24 hours and 48 hours of the beginning of the test, daphnids were observed for immobilisation and the number of organisms immobilised was recorded. Organisms that did not move following gentle agitation of the test beakers were considered immobile. The experimental setup consisted in five replicates of five neonates each, for every treatment and controls. Neonates were exposed to test solutions of triclosan and carbendazim (individually) with 50 mL of ASTM medium with no food during the 48 hours experiment (16:8h light:dark photoperiod and 20 ± 1^0C). Concentrations used were 400, 600, 800, 1000, 1200 and 1400 µg/L for triclosan and 40, 60, 80, 100, 120 and 140 µg/L for carbendazim.

2.3.2 Feeding inhibition test and post-exposure tests

Organisms with less than 24 hours old were moved to new culture aquarium and maintained at the same conditions as the main culture until 5-6 days old (corresponding to the fourth instar). The fourth instar was chosen because in this stage the feeding inhibition test (24 hours exposure plus 4 hours post-exposure) can be completed within a single moult cycle of the organism, avoiding moulting procedure interference with their feeding activity. This bioassay was based on the methodology described by McWilliam and Baird (2002). Five replicates per treatment and controls with five organisms each were used; each replicate consisted of 170 mL glass beakers containing ASTM, *R. subcapitata* at a concentration of $5x10^5$ cells/mL and the corresponding contaminant in each concentration, corresponding to a final volume of test substance of 100 mL. McWilliam and Baird (2002) performed preliminary laboratory experiments to improve this feeding bioassay with the objective of minimizing variation in baseline feeding rates and the food concentration of $5x10^5$ cells/mL was sufficient to prevent complete depletion of the food source during the feeding period (24 h). To establish that no algae growth was observed during the test, a blank set of 50 mL beakers with only algae in triplicate was also prepared for each test treatment and controls, in the same conditions as previously described, but without daphnids. The beakers were placed in a temperature controlled room at 20ºC, under darkness, during the 24 hours exposure time (corresponding to the exposure period). Following that, daphnids from each replicate were transferred into 50 mL beakers with ASTM medium, *R. subcapitata* ($5x10^5$ cells/mL) and no toxicant, and were allowed to feed for more 4 hours, also in the dark (corresponding to the post-exposure period). In the post-exposure period, five blanks with only algae and no daphnids were also prepared and maintained in the same conditions as the other beakers with daphnids, as previously describe for the exposure period. Feeding rates (cells/mL/individual/hour) were determined in accordance with the method described by Allen *et al.* (1995); algal concentration was measured using a colorimetric method at 440 nm for both exposure and post-exposure periods (Pérez *et al.*, 2011). For triclosan tested concentrations were 100, 300, 500 and 700 µg/L and for carbendazim corresponded to 100, 165, 230, 295, 360 and 425 µg/L. Concentrations of the feeding inhibition test were selected based on the immobilisation tests results and on preliminary feeding experiments. In addition, as the aim of the study was to infer on the mixture toxicity approach, a full dose-response curve is required for

calculating a better prediction of effects and therefore the concentrations used allowed this estimation.

2.3.3 Reproduction test

The reproduction test was conducted based on the OECD 211 guideline (OECD, 2008). The physico-chemical parameters pH, electrical conductivity and dissolved oxygen were recorded at the beginning, middle and end of the test. The experimental setup included ten replicates per treatment and controls with one neonate each in 50 mL glass beakers with ASTM hard water, *R. subcapitata* ($3x10^5$ cells/mL) and an organic extract (16:8h light:dark photoperiod and 20 ± 1^0C) with a final volume of test substance of 50 mL. Test solutions were renewed every other day and daphnids were fed daily. During the 21 days period, survival, the number of neonates, time to the first brood and number of broods were monitored. In addition, aborted eggs and abnormal characteristics were registered. Growth of adults (body length, in millimetres, excluding the anal spine) was determined after 21 days of exposure under a stereomicroscope (MS5, Leica Microsystems, Houston, USA). Six concentrations were tested for both compounds: for triclosan concentrations were 7.5, 15, 30, 60, 120 and 240 µg/L, and for carbendazim corresponded to 5, 20, 35, 50, 65 and 80 µg/L. These concentrations allowed a full dose-response pattern required for the mixture toxicity approach.

2.4 Mixture testing

From the individual exposure to triclosan and carbendazim 48h-LC_{50} (lethal concentration) and EC_{50} (effective concentration) values were derived (details in section 2.7) and used to design the mixture experiences. To control differences in daphnids' responses (due to sensitivity variations in organisms), single chemical exposures were performed simultaneously in each mixture test (Loureiro *et al.*, 2010). A full factorial design was used in the immobilisation test (Fig. 2.1 SD a), by crossing all the concentrations used in the single exposure assessment. In the feeding inhibition and reproduction tests, a fixed ratio design was conducted to avoid mortality at higher chemical combinations (Fig. 2.1 SD b and c). In the feeding inhibition and reproduction tests, EC_{50} values obtained from single exposures were used to calculate toxic units (Fig. 2.1 SD), and

the toxic unit sum (∑TUs) never exceeded 2 to prevent mortality. For the feeding inhibition and reproduction experiments, concentrations were based on expected toxic strengths of 0.375 (0.125+0.25; 0.25+0.125), 0.5 (0.125+0.375; 0.25+0.25; 0.375+0.125), 0.75 (0.125+0.625; 0.25+0.5; 0.375+0.375; 0.5+0.25; 0.625+0.125), 1 (0.125+0.875; 0.25+0.75; 0.375+0.625; 0.5+0.5; 0.625+0.375; 0.75+0.25; 0.875+0.125), 1.5 (0.75+0.75 ; 1+0.50; 0.50+1), 1.75 (1+0.75; 0.75+1) and 2 (1+1) toxic units ($TU_{Chemical\ 1}$ +$TU_{Chemical\ 2}$).

2.4.1 Immobilisation, feeding inhibition and reproduction tests

Bioassays described above were also carried out with small adaptations mainly by decreasing the number of replicates used, to increase the number of mixture treatments, increasing therefore covering the most of the response surface. This has been argued to increase both reliability and power of the analysis, as the response surface analysis is based on a regression model (Loureiro *et al.*, 2010).

In the immobilisation test three replicates of five neonates each were used; each replicate had a final volume of 50 mL of the test substance. Chemical single exposure were carried out simultaneously with concentrations of 400, 600, 800, 1000 and 1200 µg/L for triclosan and of 20, 70, 120, 170 and 220 µg/L for carbendazim and the combinations of both, by using a full factorial design with a total of 25 treatments.

The experimental design for the feeding inhibition test included, simultaneously, a single evaluation of each compound and a set of binary mixtures (23 combinations). In this mixture experiment we used one replicate for each treatment and each replicate contained five daphnids (5-6 days old, corresponding to the fourth instar); in the 24 hours exposure period each replicate had a final volume of 100 mL of the test substance and in the 4 hours post-exposure period each replicate had 50 mL of the test substance (McWilliam and Baird, 2002). In single experiments, concentrations were 200, 350, 500 and 650 µg/L and 150, 225, 300 and 375 µg/L for triclosan and carbendazim, respectively. In combined experiments (after the TU calculation), concentrations ranged from 60 to 500 µg/L and 30 to 260 µg/L for triclosan and carbendazim, respectively (Fig. 2.1 SD b).

In reproduction experiments, survival, number of neonates produced per organism, time to the first brood and number of broods were monitored during 21 days; each control and concentration were conducted with one replicate with one daphnid each; each replicate had a final volume of 50 mL of the test substance In single chemical experiments,

concentrations applied in the reproduction test were 17.5, 35, 70, 140 and 280 µg/L for triclosan and for carbendazim were 4, 8, 16, 32 and 64 µg/L; in combined experiments, after the TU calculation, concentrations ranged from 25.75 to 206 µg/L for triclosan and from 2.89 to 23.12 µg/L for carbendazim (Fig. 2.1 SD c).

2.5 Comet assay: single and mixture testing

D. magna juveniles were exposed to concentrations of 5, 20 and 25 µg/L of carbendazim, corresponding to the NOEC (no observed effect concentration), LOEC (lowest-observed-effect concentration) and EC_{50} values for carbendazim in the reproduction test. For triclosan exposure, daphnids were exposed to 120, 160 and 206 µg/L of triclosan, where the extremes concentrations correspond to the NOEC and EC_{50} of triclosan in the reproduction tests, however the middle concentration of triclosan was chosen considering the reproduction results. Mixture exposures of 120 µg/L of triclosan plus 5 µg/L of carbendazim, 160 µg/L of triclosan plus 20 µg/L of carbendazim, and 206 µg/L of triclosan plus 25 µg/L of carbendazim, were also tested in *D. magna* juveniles with less than 24 hours. Mixture exposures were conducted at the same time as individual ones. These concentrations were chosen considering the results from the previous tests performed. Under these concentrations daphnids were not expected to die and concentrations where: no effects were observed, low effect levels were observed or where a significant impairment was observed in terms of reproduction (the most sensitive parameter used in the present study) were included in this set up. This was mainly based on the knowledge that some effects may not be detected using individual endpoints (*e.g.* feeding activity or reproduction), however, at lower concentrations a subcellular effect, like DNA damage, may occur (Jha, 2008). In addition, the concentration range used enable to derive EC_{50} values needed for the mixture analyses using the MIXTOX tool.

At all experiments, four replicates with fifteen juveniles of *Daphnia* (<24h) each, were used for each control and concentrations. Each replicate consisted of one glass beaker containing 150 mL ASTM (plus contaminants in treatment situation). After 24 hours exposure, organisms were collected and pooled for the comet assay as described by Nogueira *et al.* (2006). Positive controls were performed by using daphnid cells previously exposed to hydrogen peroxide (H_2O_2). To prevent UV induced DNA damage, assays were conducted under yellow light. Organisms were placed in 1.5 mL *Eppendorfs* containing 1

mL of phosphate-buffered saline (PBS) with 10% dimethyl sulfoxide (DMSO) and 20 µM ethylene diamine tetra-acetic acid (EDTA). Organisms were gently disintegrated mechanically with an appropriate spatula (carapaces were removed when possible). *Eppendorfs* were centrifuged (200 *g*) during 10 minutes at 4ºC. The supernatant was gently removed. From this suspension of cells, 10 µL were transferred to *Eppendorfs* containing 0.5% of low melting point agarose, at 37ºC. The mixture was spread on the microscope glass slides, and then the cover slips were applied. Slides were placed on ice for 10 minutes. Subsequently, slides (without coverslips) were placed, for at least 1 hour, in a solution of 10 mM Tris-HCl, 100 mM EDTA, 2.5 M NaCl, 10% DMSO, 10% Triton X-100, pH 10, for cell lysis. Slides were left for 15 minutes in the electrophoresis tank filled with a 10 M NaOH, 200 mM Na_2 EDTA solution (to allow DNA denaturation and unwinding) before electrophoresis took place. For the electrophoresis, an electric current of 300 mA (30 Volts) was applied for 10 minutes. To neutralize, slides were washed with 0.4M Tris-HCl (pH=7.5). Slides were dehydrated with absolute ethanol 100% for 10 seconds (left to dry for 1 day in the dark).

Slides were stained with 100 µL ethidium bromide (20 µL/mL), before analysis in a fluorescent microscope (Olympus BX41TF, China) at 400x magnification. The slide reading was done randomly, to avoid bias on the results and one hundred cells per slide were examined. DNA damage was visually scored: cells were scored on a 0 to 4 scale, as described by Duthie and Collins (1997). Fig. 2.2 SD represents a comet type scale in daphnid cells. Type 0 represents no DNA damage, type 1 and 2 represent mild to moderate damage, respectively, and type 3 and 4 represent extensive DNA damage. Therefore, the total score for 100 cells could range between 0 (all comets with no damage) to 400 (all comets with maximal damage). A percentage of DNA damage (for the single experiments) and percentage of no DNA damage (for the MIXTOX analysis) was calculated.

2.6 Statistical Analysis

2.6.1 Single chemical testing

Differences between organisms exposed to the negative control and to the solvent control were checked using a t-test (Systat Software Inc., 2008).

Data from the immobilisation tests of *D. magna* were analysed using a probit analysis and a 48h-LC_{50} value was derived (Minitab Version 14.0, 2003).

The statistical analysis for the sub-lethal parameters (feeding activity, reproduction and DNA damage) was performed with SigmaPlot v11.0 software (Systat Software Inc., 2008). To detect significant differences between treated groups and control values a one-way analysis of variance (ANOVA) was used. The normality of the data was tested using the Kolmogorov-Smirnov test. A Dunnett's test was carried out when differences were obtained in data that followed a normal distribution. If data were not normally distributed and data transformation did not correct for normality, a Kruskal-Wallis test was also used and the multiple comparisons Dunn's Method. The 50% effective concentration (EC_{50}) values were calculated using a nonlinear regression with a logistic/sigmoid function using always the one with better adjustment (Systat Software Inc., 2008).

2.6.2 Mixture testing

The toxic effects of the mixtures were analysed by comparing the obtained effects with the expected mixture effects (based on single exposures) using the reference conceptual model, IA, in the MIXTOX tool described by Jonker *et al.* (2005). The mathematical formulation of the IA model is based on probability of responses and is expressed as:

(Equation 3) $Y = \mu\, max \prod_{i=1}^{n} qi\ (Ci)$

Where *Y* denotes the biological response, C_i is the concentration of chemical *i* in the mixture, *qi (Ci)* the probability of non-response, μ_{max} the control response for endpoints and $\prod$ the multiplication function.

Deviations from this model were modelled for synergism or antagonism, dose ratio and dose level dependencies by adding two extra parameters: *a* and *b*; its biological interpretation is described in Table 2.1 SD and further explanation can be found in Jonker *et al.* (2005). The method of maximum likelihood was used to fit the data and the fundamental procedure to minimize the Sum of Squared Residuals (SS) was used by running the Solver Function in Microsoft® Excel.

3. *Results*

3.1 Chemical analysis

Chemical analysis showed that the carbendazim concentration decreased over time, with a decay rate (K_0) of 0.03/hour (SE=0.005), and only 18% of the initial concentration remained after 48h. Regarding triclosan, the obtained decay rate (K_0) was 0.06/hour (SE=0.010), and after 48h of the initial concentration only 1.3% of triclosan concentration remained.

3.2 Single chemical testing

The validation of the immobilisation tests, as established by the OECD 202 guideline, was fulfilled (OECD, 2004). Also, the reproduction tests was valid as established by the OECD 211 guideline, with the parent animals in the control showing less than 20% of mortality and the mean number of live offspring per *Daphnia* at the end of the test was higher than 60. Regarding physico-chemical properties: pH, dissolved oxygen concentrations and conductivity (Table 2.2 SD), all parameters were also in accordance with the OECD 211 guideline (OECD, 2008).

In all bioassays, there were no differences between the negative and the solvent controls at the 5% level. Therefore all comparisons between chemical treatments and the control group were carried out with the solvent control.

The EC_{50} values obtained for each single chemical exposure for triclosan and carbendazim are summarized in Table 2.1. Values from the single chemical bioassays were used to calculate the TU values for the experimental setup of the mixture exposure but also to compare accuracy of results from the single exposures used (simultaneously) in the mixture approach.

Acute exposures to triclosan and carbendazim derived 48h-LC_{50} values of 856.8 µg/L (SE=28.4) and 87.6 µg/L (SE=5.6), respectively (Table 2.1).

The feeding activity upon exposure to triclosan and carbendazim followed a dose-response relationship (Fig. 2.1). The EC_{50} value calculated for triclosan was of 549.3 µg/L (SE=19.2, r^2=0.88), still followed by a similar effect regarding the 4h post-exposure, where an EC_{50} of 478.0 µg/L (SE=33.5, r^2= 0.75) was derived (Table 2.1). For carbendazim, EC_{50}

values derived were of 325.6 µg/L (SE=90.4, r^2=0.71) and 176.5 µg/L (SE=9.9, r^2=0.93) for the 24h exposure and 4h post-exposure, respectively (Table 2.1).

Table 2.1. 48h- LC50/EC50 (µg/L) (with standard error (SE) values between brackets), NOEC (µg/L) and LOEC (µg/L) for the endpoints: immobilisation, feeding inhibition and reproduction, obtained from the exposure of *Daphnia magna* to triclosan and carbendazim.

Triclosan

Test	Parameter	LC/EC_{50} value (SE)	r^2	NOEC	LOEC
Immobilisation test	**Immobilisation**	856.8 (28.4)	-	-	-
Feeding inhibition test	**Feeding rate 24h**	549.3 (19.2)	0.88	300	500
	Feeding rate 4h	478.0 (33.5)	0.75	500	700
Reproduction test	**Number neonates**	206.2 (10.7)	0.88	120	240

Carbendazim

Test	Parameter	LC/EC_{50} value (SE)	r^2	NOEC	LOEC
Immobilisation test	**Immobilisation**	87.6 (5.6)	-	-	-
Feeding inhibition test	**Feeding rate 24h**	325.6 (90.4)	0.71	230	295
	Feeding rate 4h	176.5 (9.9)	0.93	<100	100
Reproduction test	**Number neonates**	23.2 (2.2)	0.88	5	20
	Aborted eggs	28.8 (7.4)	0.62	20	35

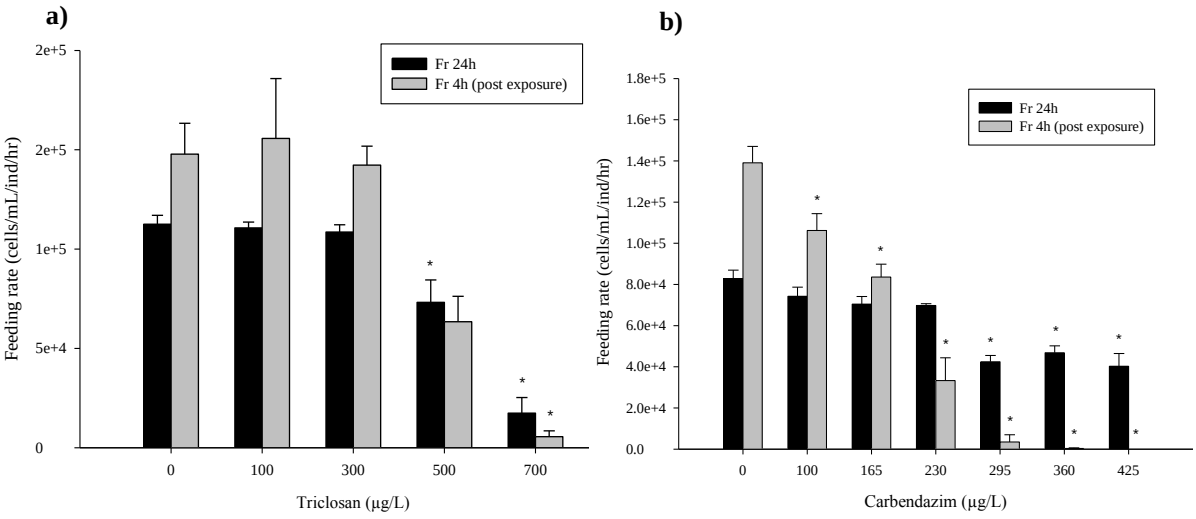

Figure 2.1. Feeding rate (cells/mL per daphnia per hour) of *Daphnia magna* (n=5, 5 replicates) exposed to a) triclosan and b) carbendazim. Data is expressed as mean values and standard error. Dark bars represent the feeding rate (Fr) after a 24h exposure period and grey bars represent the feeding rate (Fr) during the 4h post-exposure. (* $p<0.05$, Dunnett's Method, in comparison with the control).

In the reproduction test, 100% mortality was observed after 16 days of exposure in the two highest concentrations of carbendazim (65 and 80 µg/L; data not shown) and therefore these two concentrations were excluded from the reproduction output analysis. The mean number of neonates produced per daphnia decreased significantly with increasing concentrations of carbendazim (ANOVA, $F_{4,32}$=61.72 p<0.001) (Fig. 2.2 b). However, for triclosan, only the highest concentration (240 µg/L) induced a significant decrease of the number of neonates when compared to the control (ANOVA, $F_{6,59}$=101.6 p<0.001) (Fig. 2.2 a). For carbendazim the (mean) number of aborted eggs increased with carbendazim concentrations, as shown in Fig. 2.2 b. The calculated EC_{50} values for the effects on reproduction for daphnids exposed to carbendazim were 23.2 µg/L (SE=2.2, r^2=0.88) for the number of neonates per female and 28.8 µg/L (SE=7.4, r^2=0.62) for the number of aborted eggs, with a NOEC and a lowest-observed-effect concentration (LOEC) of 5 µg/L and 20 µg/L, respectively, for the number of neonates (Table 2.1).

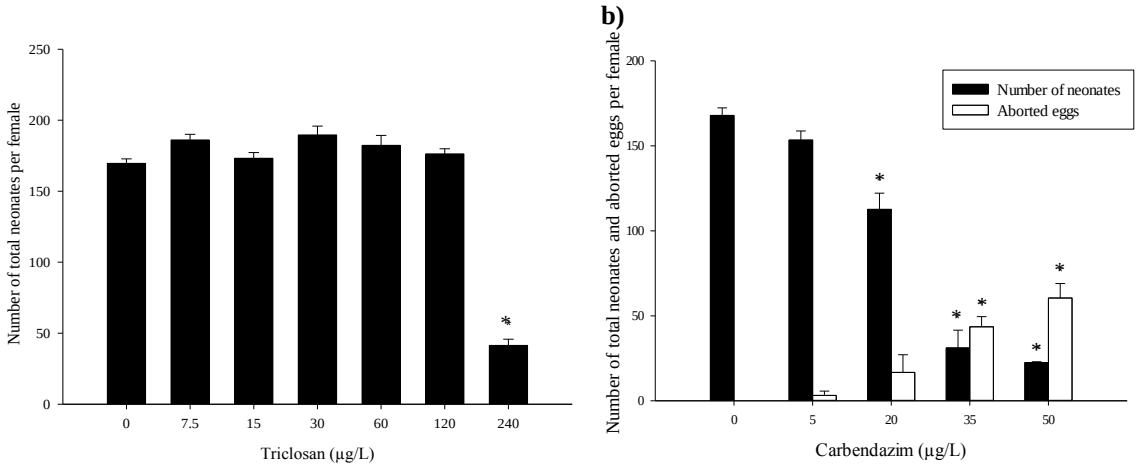

Figure 2.2. Reproduction effort during a 21 days exposure period of *Daphnia magna* (n=1, 10 replicates) to a) triclosan and b) carbendazim. Black bars refer to the number of neonates and white bars to the number of aborted eggs. Data is expressed as mean values and standard error (* p<0.05, Dunnett's and Dunn's (only for aborted eggs data) Method, in comparison with the control).

The body length of adult daphnids after the 21 days of exposure to triclosan was similar within all concentrations when compared to the control. For carbendazim, the concentration of 35 µg/L was the only one where daphnids length decreased significantly when compared to the control (Kruskal–Wallis one-way ANOVA, H=14.91, DF=4;

p=0.005; Dunn's Method p<0.05) (Fig. 2.3). The calculated EC_{50} value for the reproduction (number of neonates per female) of daphnids exposed to triclosan was 203.2 µg/L (SE=10.7, r^2=0.89), with a NOEC and LOEC of 120 µg/L and 240 µg/L, respectively (Table 2.1).

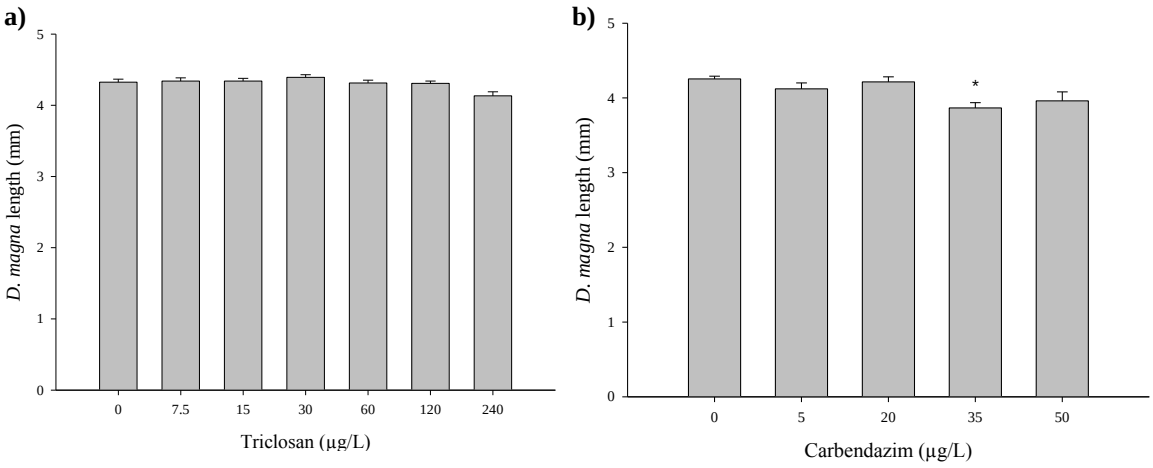

Figure 2.3. Body length (mm) of *D. magna* (n=1, 10 replicates) after 21 days exposure to a) triclosan and b) carbendazim. Data is expressed as mean values and standard error (*p<0.05, Dunnett's and Dunn's Method, for triclosan and carbendazim, respectively, in comparison with the control).

3.3 Mixture testing

The combined toxicity of triclosan and carbendazim in the immobilisation test was significantly adjusted to the IA model (Table 2.2). Continuing the nested framework for assessing potential deviations, the dose level deviation pattern showed to present the best fit (Table 2.2). Regarding the isobolograms (Fig. 2.4 a1), and also the derived positive parameter *a* means that there was an antagonism at low doses of both chemicals and synergism at high doses; and parameter *b* was lower than one, meaning that the change from antagonism to synergism would occur at higher concentrations than the tested ones. Therefore synergism was not observed in the isobologram and the main pattern for this endpoint was antagonism (see Table 2.1 SD).

In the feeding inhibition of *D. magna* (24h exposure), the IA model fitted our data significantly with no further improvement by adding parameters for deviations, showing a

pattern for additivity on effects (Table 2.2, Fig. 2.4 b1). In the 4h post-exposure period of this test, the same additivity was observed (Table 2.2, Fig. 2.4b'1).

For the reproduction data (total number of neonates per female) the MIXTOX results showed that the conceptual model (IA) adjusted well and significantly to this data (Table 2.2, Fig. 2.4c1), with no significant deviations.

Table 2.2. MIXTOX analysis of the combinations of triclosan and carbendazim to immobilisation, feeding inhibition (24h exposure and 4h post-exposure), reproduction and % of no DNA damage of *Daphnia magna*.

	Endpoint: **Immobilisation**			
	Independent Action			
	IA	S/A	DR	DL
r^2	0.76	0.79	0.80	0.82
SS	76.58	66.71	66.63	57.56
***p*(*F*-test)**	**$1.24x10^{-52}$**	-	-	-
$p(\chi^2)$	-	**0.0017**	0.78	**0.002**
max	0.98	0.98	0.98	0.98
a	-	2.69	3.36	0.017
b	-	-	-1.18	-251.80
	Endpoint: **Feeding inhibition 24h exposure**			
	Independent Action			
	IA	S/A	DR	DL
r^2	0.79	0.81	0.82	0.81
SS	$6.64x10^{9}$	$5.95x10^{9}$	$5.70x10^{9}$	$5.92x10^{9}$
***p*(*F*-test)**	**$8.79x10^{-9}$**	-	-	-
$p(\chi^2)$	-	0.06	0.09	0.16
max	128455.4	131888.1	133293.3	130086.6
a	-	2.09	-0.19	3.27
b	-	-	5.24	0.73
	Endpoint: **Feeding inhibition 4h post-exposure**			
	Independent Action			
	IA	S/A	DR	DL
r^2	0.40	0.40	0.40	0.40
SS	$5.28x10^{10}$	$5.28x10^{10}$	$5.28x10^{10}$	$5.28x10^{10}$
***p*(*F*-test)**	**0.0066**	-	-	-
$p(\chi^2)$	-	0.99	0.99	0.99
max	201182.7	201015.9	201015.9	201015.9
a	-	0	0	0
b	-	-	0	2
	Endpoint: **Reproduction**			
	Independent Action			
	IA	S/A	DR	DL
r^2	0.79	0.79	0.83	0.82
SS	6485.96	6485.29	5403.94	5657.28
***p*(*F*-test)**	**$1.51x10^{-7}$**	-	-	-
$p(\chi^2)$	-	0.96	0.08	0.15
max	176.15	176.14	176.47	175.18
a	-	-0.18	103.39	36.67
b	-	-	-142.75	3.48
	Endpoint: **% of no DNA damage**			
	Independent Action			
	IA	**S/A**	DR	DL
r^2	0.88	0.89	0.92	0.90
SS	755.99	712.07	481.01	638.45
***p*(*F*-test)**	**$2.95x10^{-14}$**	-	-	-
$p(\chi^2)$	-	0.14	**0.00023**	**0.04**
max	72.22	73.79	71.71	73.18
a	-	0.57	-5.50	-3.29
b	-	-	16.07	1.92

r^2 represents the maximum likelihood test; SS represents the sum of squared residuals; *p*(*F*-test) represents the result of the likelihood ratio test; $p(\chi^2)$ represents the outcome of the likelihood ratio test; max represents the control response, *a* and *b* represents the additional parameters of the function; IA represents the independent action model; S/A represents synergism/antagonism; DR represents the dose ratio dependence; DL represents the dose level dependence.

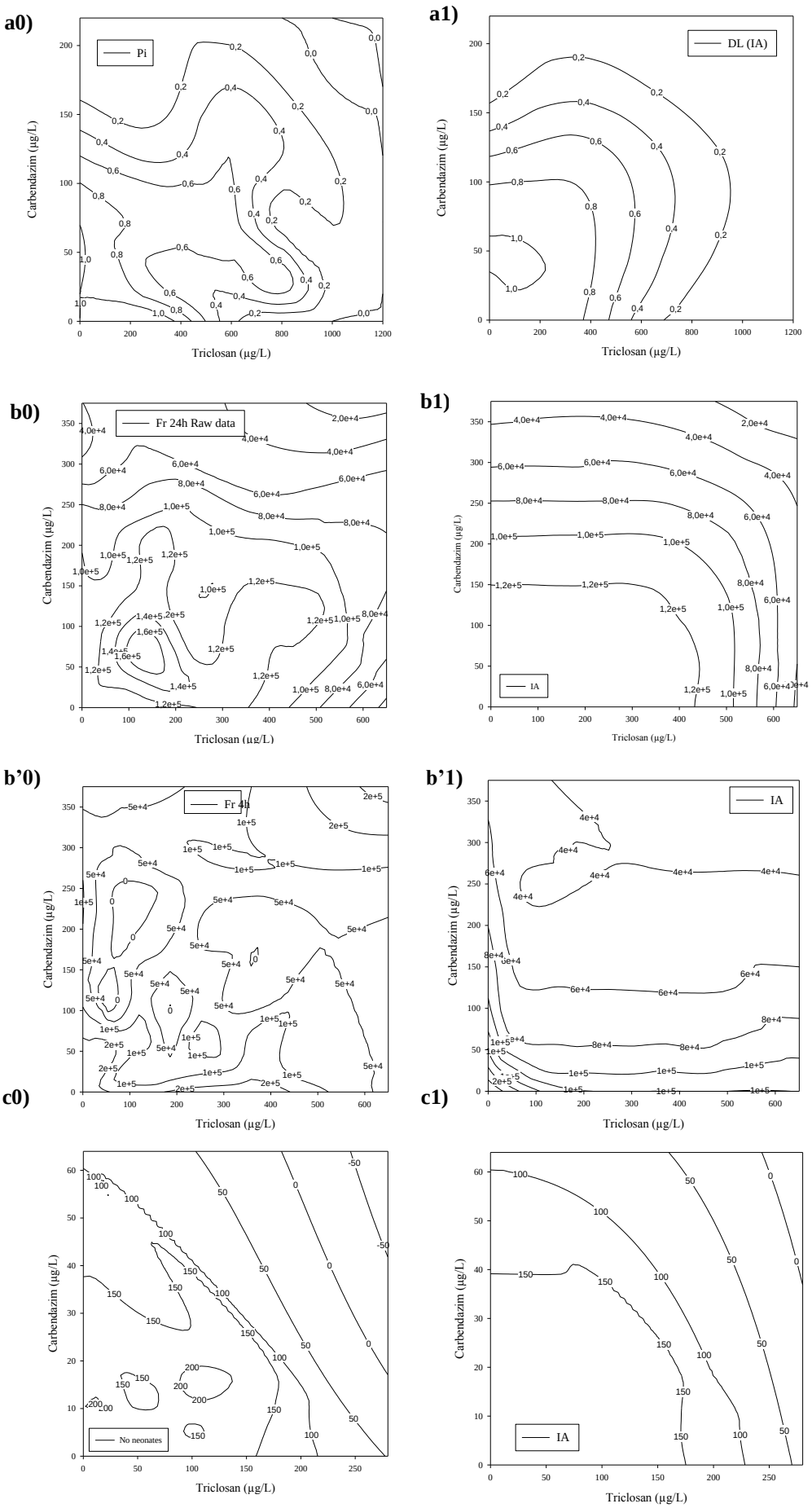
a0)
Pi
a1)
DL (IA)
b0)
Fr 24h Raw data
b1)
IA
b'0)
Fr 4h
b'1)
IA
c0)
No neonates
c1)
IA
Carbendazim (µg/L)
Triclosan (µg/L)

Figure 2.4. Concentration-response relationship for the binary mixture of triclosan and carbendazim (2D isobolic surfaces). a) Survival of *Daphnia magna* (Pi) (n=5, 3 replicates)*:* a0) observed data a1) showing a DL deviation from the IA model b) Feeding rate of *D. magna* (cells/mL/ind/hr) at 24h (n=5, 1 replicate): b0) observed data b1) showing no deviation to the IA model b') Feeding rate of *D. magna* (cells/mL/ind/hr) at 4h post-exposure (n=5, 1 replicate): b'0) observed data; b'1) showing no deviation to the IA model c) Reproduction of *D. magna* (number of total neonates per female after 21 days of exposure) (n=1, 1 replicate): c0) observed data; c1) showing no deviation to the IA model.

3.4 Comet assay: single and mixture testing

Both compounds and the mixture showed an increase in DNA damage with increasing concentrations, comparing with the control situation (Fig. 2.5). For both chemicals the percentage of damage was significant at all the concentrations tested (Dunnett's Method $p<0.05$). The lowest concentration used in the comet assay corresponds to the NOECs from the reproduction tests, where no effects on the reproduction output were observed. However, these carbendazim and triclosan levels/concentrations already induced a significant increase in DNA damage.

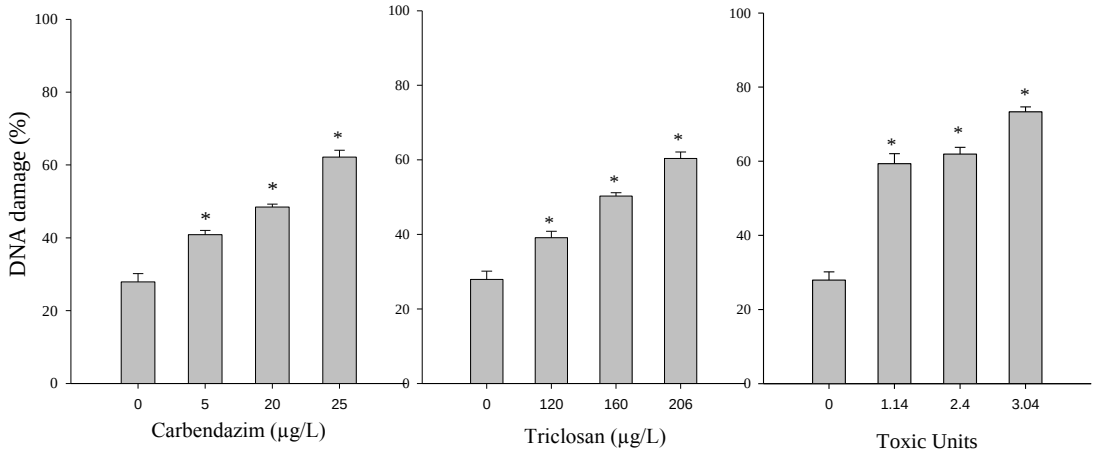

Figure 2.5. DNA damage (%) in *D. magna* cells exposed to carbendazim, triclosan and mixture of both (Toxic Units) (n=15, 4 replicates) (* $p<0.05$, Dunnett's Method, in comparison with the control).

Regarding mixture toxicity effects, in order to get a more accurate analysis of the dataset, the percentage (%) of DNA damage was converted into % of cell viability (% of no damage) in *D. magna*, in order to get a dose-response curve with decrease on results,

when concentrations were increased. From the MIXTOX model fit (Table 2.2, Fig. 2.6) it was observed that the data was well modelled by the DR deviation (Table 2.2). In the DR deviation, when testing two compounds in a mixture, deviations will depend on the mixture composition, and the predominance of the chemical which is inducing effects, *i.e.*, synergism is induced mainly by one of the chemicals in the binary mixture, and the other chemical is responsible for the opposite pattern, antagonism (Jonker *et al.*, 2005; Loureiro *et al.*, 2010). In our study, for the % of no DNA damage, the biological interpretation of the derived extended parameters *a* and *b* showed that synergism was mainly driven by triclosan and carbendazim was the main responsible for the increase of cell viability (% of no damage) (antagonism) (see Table 2.1 SD). The selection of this deviation is supported by the r^2 value and mostly by the SS, which are the lowest comparing with all the others deviations in the IA model.

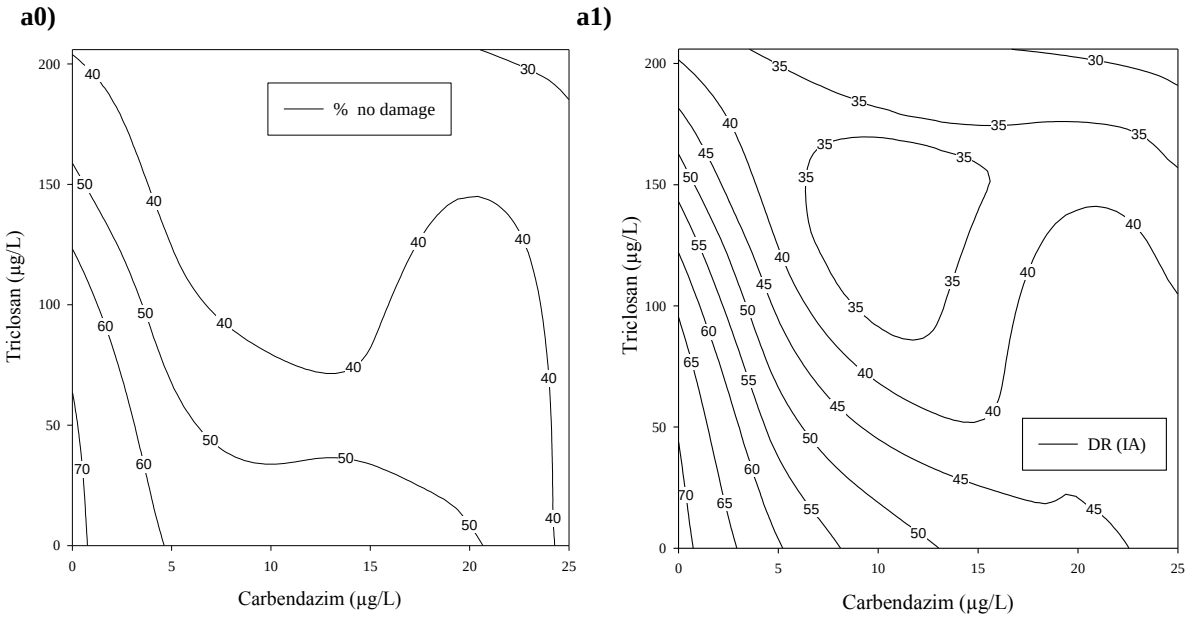

Figure 2.6. Concentration-response relationship for the binary mixture of triclosan and carbendazim (2D isobolic surfaces) (n=15, 4 replicates) a) % of no DNA damage on *D. magna* cells a0) observed data a1) showing a DR deviation from the IA model.

4. Discussion

4.1 Single chemical testing

In the immobilisation tests, and from the 48h-LC_{50} values derived, carbendazim presented to be approximately ten times more toxic than triclosan. Previous studies have reported 48h-LC_{50} value of 390 µg/L for triclosan (Orvos *et al.*, 2002) and 110, 157 and 350 µg/L for carbendazim (Ferreira *et al.*, 2008; U.S.EPA, 2000) showing a high variability in the results depending on the study.

In the single exposure to carbendazim, an EC_{50} value of 325.6 µg/L was derived for the feeding inhibition (24h exposure) endpoint. Feeding activity of *D. magna* exposed to this compound was already investigated in the work of Ribeiro *et al.* (2011) and Ferreira *et al.* (2008), where EC_{50} values for the 24 hours exposure were lower: 179.87 µg/L and 97.54 µg/L, respectively. Regarding triclosan, and to our knowledge, the effects of triclosan on the feeding activity of *D. magna* have not been reported in literature. In the post-exposure period, when daphnids were moved to clean medium (with no contaminant), there was an increase on feeding rates (comparing with 24h exposure) in the two first concentrations for both triclosan and carbendazim (Fig. 2.1). This pattern was also observed by Ferreira *et al.* (2008), which suggests that this increase on feeding rates upon exposure could indicate compensation induced by a previous chemical stress. However, daphnids from the highest concentrations of both compounds may not have recovered from the chemical exposure, having still a decrease on feeding rates in the post-exposure period (Fig. 2.1). McWilliam and Baird (2002) verified that some compounds also produced feeding depression during this period. Therefore, in risk assessment, adding this kind of information on post-exposure effects can be considered ecologically relevant, as it highlights the ability of organisms to recover after short periods of exposure (McWilliam and Baird, 2002).

In the reproduction test with carbendazim, there was an increase in the number of aborted eggs with increasing concentrations (Fig 2.2b). This pattern was also observed by Ribeiro *et al.* (2011) and it is probably related to the described mode of action of carbendazim in fungi, which inhibits the mitosis, and its teratogenic effects have been reported along the years (Davidse, 1977). In the study of Ribeiro *et al.* (2011), after 21 days exposure an EC_{50} value of 40.05 µg/L was determined for *D. magna*, which is in the

same order of magnitude from the one in the present study (EC_{50}= 23.2 µg/L), although two times higher. In the 70's, fungicides belonging to the same group of carbendazim: benzimidazoles showed effects on the reproductive capacity of *D. magna*, with an EC_{50} value of *ca.* 20 µg/L of BCM (methyl benzimidazole-2-yl carbamate) (Canton, 1976). Since the 90's toxicity of carbendazim has been studied, and van Wijngaarden et al (1998) studied the effect of Derosal (a formulation containing carbendazim) in some aquatic invertebrates, including *D. magna*; this organism was relatively sensitive with a NOEC value (for the number of neonates per female) of 25.8 µg/L. For the endpoint reproduction of *D. magna* as well, Van den Brink *et al.* (2000) reported a 28 days NOEC and EC_{50} value of 33 and 37 µg/L, respectively. Other effects that might be related to effects on reproduction in these crustaceans are the production of male daphnids. This phenomena was observed upon an exposure of 40 µg/L of carbendazim to the crustacean *Moina micrura* which lead to the production of males with abnormal antennules (Miracle *et al.*, 2011).

For triclosan the calculated EC_{50} value (regarding the number of neonates per female) was 203.2 µg/L. Some studies tested the effects of triclosan on the reproduction of *Daphnia* (Flaherty and Dodson, 2005; Orvos *et al.*, 2002), however, to our knowledge no EC_{50} value was derived. Tatarazako *et al.* (2004) tested the effect of triclosan on the reproduction of the crustacean *Ceriodaphnia dubia*, and an IC_{50} (inhibiting concentration with a reduction of 50% in reproduction) similar to our study was obtained (220 µg/L).

In the single exposure to carbendazim, daphnids feeding inhibition occurred at higher concentrations than those impairing reproduction. These could be related to the age of the organisms, as in the reproduction test, daphnids had less than 24h when exposure started, and 5 to 6 days when they were exposed to the chemical in the feeding inhibition tests, possibly being less sensitive. In addition, the exposure in the feeding inhibition test lasted for 24h and in the reproduction test the exposure was longer (21 days).

Other organisms have been used to test the toxic effects of both compounds and different degrees of sensitivity were found. For the algae *Tetrahymena pyriformis*, carbendazim have showed to be far less toxic with an EC_{50} value of 6380 µg/L, however carbendazim was more toxic to the fish *Ictalurus punctatus* with an EC_{50} value of 10 µg/L (RIVM, 2008). For triclosan, an LC_{50} value of 1700 µg/L was found with a 96 hours

exposure using the fish *Oryzias latipes* and for the algae *Anabaena flos aquae* triclosan was highly toxic with an EC_{50} value of 1.6 µg/L (Dann and Hontela, 2011).

The genotoxicity of triclosan and carbendazim was evaluated in daphnid cells, where it was witnessed that both compounds caused an increase in DNA damage with increasing concentrations (comparing with the control) (Fig. 2.5). This was observed even at NOEC levels derived for the reproduction tests, showing that the DNA damage can be considered in this study as an early warning endpoint, regarding effects. Over the last years, genotoxic effects were reported for both compounds in different organisms. JanakiDevi and colleagues (2013) determined that in the marine invertebrate *Donax faba*, different concentrations of carbendazim induced higher DNA damage comparing with the control samples. In the zebra mussel, *Dreissena polymorpha*, and in the algae, *Closterium ehrenbergii*, triclosan induced DNA damage as well (Binelli *et al.*, 2009; Ciniglia *et al.*, 2005). The DNA strand breaks caused by genotoxic compounds may give rise to chromosomal aberrations that can promote cell death and may be related with mitotic anomalies, and consequently lead to acquisition of DNA damage (Ganem and Pellman, 2012; Jha, 2008). This is probably related with the presence of aborted eggs upon carbendazim exposure both in the present study and in another study described by Ribeiro *et al.* (2011), considering that the mode of action of carbendazim on fungi is related with mitosis. DNA damage occurred at exposures of 5 µg/L of carbendazim and there are reported environmental concentrations in surface waters of 4.5 µg/L of carbendazim (in the basin of the Traiguén River, Chile) (Palma *et al.*, 2004). Therefore, this study highlights the importance of lower organizational level parameters to understand mechanisms but also to be used as early warning tools (no effects occurred at the individual level in this specific concentration, while DNA damage was already occurring).

There are not many Maximum Allowable Concentration (MAC) available in legislation regarding triclosan. The Russian Federation established a MAC for carbendazim in surface waters of 0.1 mg/L (WHO, 1993). Considering the results of our study, where carbendazim concentrations below 0.1 mg/L (100 µg/L) caused effects at the reproduction and at subcellular level (DNA damage), this might represent a low protection level, and therefore risk. An identical hazard situation might occur for triclosan, as high triclosan concentrations, 6000-14000 µg/L, were detected in a USA river receiving a

treated wastewater discharge from a manufacturing plant. Moreover in Spain primary effluents of an urban WWTP presented a concentration of 269 µg/L of triclosan (Jungclaus *et al.*, 1978; NICNAS, 2009).

4.2 Mixture testing

Considering the possible effects of the mixture of triclosan and carbendazim in aquatic systems and the unawareness of the specific molecular mode of action of triclosan and carbendazim on *D. magna* as stressed before, the IA model was tested and deviations from additivity occurred in some of the case studies (Table 2.2). An additive effect (IA model) was observed for the feeding inhibition and on the reproduction data. Other studies on the effects of insecticides binary mixtures to *D. magna* have demonstrated that both mixture conceptual models (CA and IA) can explain data equally well, however, they suggested that the CA was the most conservative model to predict mixture effects (Syberg *et al.*, 2008) and potentially the easiest to use for legislators. For the case of triclosan, DeLorenzo and Fleming (2008) tested the mixture of triclosan with the pharmaceutical fluoxetine (marketed as Prozac®) on the 96h growth test with the algae *Dunaliella tertiolecta* and an additive effect was also observed.

In the present study an additive effect was observed in the feeding inhibition test (24h exposure). Ferreira *et al.* (2008) tested the effect of the binary mixture of carbendazim and cadmium and the combination of carbendazim and the environmental variable - dissolved oxygen, on the feeding activity (24h exposure) of *D. magna*, and they found an antagonistic pattern for both combinations. In the immobilisation test antagonism was observed at low doses of the chemical mixture of triclosan and carbendazim (in the DL deviation). Opposite effects were reported for carbendazim and copper mixture in the reproduction of *Caenorhabditis elegans*, where synergism was observed at low doses and antagonism at high doses of those chemicals (Jonker *et al.*, 2004).

Despite this additive effect, when looking at the DNA damage in daphnid cells, synergism (in the DR deviation) was also observed and it was mostly related to triclosan. Synergism is considered the worst scenario because it means that the toxic effects of the mixture are more severe than expected regarding the individual chemical toxicity, and there is an augmentation of toxicity (Jonker *et al.*, 2005; Santos *et al.*, 2011a). Since triclosan interferes with the fatty acid biosynthesis in bacteria, the majority of the studies

that are available in the literature are performed in microorganisms. In *Vibrio fischeri* a synergistic effect was observed in the presence of triclosan jointly with LAS (linear alkylbenzene sulfonates), which is another compound found in domestic wastewaters (Farré *et al.*, 2008). In other bacteria, such as *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Escherichia coli*, synergism was found in the antibiofilm activity when these bacteria were exposed to triclosan and DispersinB® (Darouiche *et al.*, 2009). The same pattern (synergism) was observed in the *Salmonella Typhimurium* biofilms, when exposed to triclosan and the antibiotic ciprofloxacin (Tabak *et al.*, 2009). Therefore, this result for synergism may be indicative of the actual underestimation of the risk to aquatic communities exposed to these two compounds and seems somehow related to the presence of triclosan. In the present study DNA damage provided an output considerably important as it showed effects at NOEC levels using standardised tests (reproduction), but also in the mixture approach. When looking at the currently used risk evaluations those are mainly focused on individual toxicity of compounds and disregard their potential interaction inside organisms. The comet assay results provided also an input showing DNA damage higher than expected if one regards the toxicity of both compounds acting singly.

Interactions between compounds can influence several processes in organisms, including bioavailability, uptake, metabolization, excretion, *etc*. One possible explanation for the synergy in the simultaneous presence of carbendazim and triclosan could be probably related with changes in the metabolic enzyme activities. One similar example was observed with the lipophilic insecticide (pyrethroid) that enhanced the toxicity when mixed with the azole fungicide (prochloraz). Here the azole inhibited the metabolization of the pesticides in daphnids, increasing the overall toxicity (Cedergreen, 2014).

From the results of the present study, we derived different patterns of toxicity for this chemical mixture for different endpoints, including additivity, synergism and antagonism, showing that chemicals can also interact among them in the organisms (Loureiro *et al.*, 2010). As shown in our work, even within the same organism (*D. magna*) different evaluated endpoints provided different outputs regarding mixture toxicity. This was already observed in other studies, where effects on the same organism varied depending on the endpoint chosen (Loureiro *et al.*, 2010; Santos *et al.*, 2011b; Turgut and Formin, 2002). This is possibly related to the chosen endpoint, which is interconnected with the chemical

mode of action, but also with the potential mechanism of interaction between chemicals inside organisms.

5. Conclusions

D. magna showed to be more sensitive to carbendazim than to triclosan. Through the comet assay it was observed that both compounds caused DNA damage in daphnids cells even at NOEC levels for reproduction, with carbendazim showing higher toxicity. In the mixture toxicity all possible outcomes were observed: non-interaction or additivity, synergistic and antagonistic patterns. The reference model, IA, explained the results from feeding inhibition and reproduction data, and on the other hand dose level or dose ratio dependencies were observed for the other endpoints (immobilisation and DNA damage). As a worst case scenario, synergism was attained using DNA damage as endpoint, mainly induced by triclosan.

Usually risk assessment is carried out by focusing on the toxicity of single chemicals, however, and as we know, in the environment compounds appear as complex mixtures. Considering these, mixture toxicity studies will provide more and useful information to predict risk more accurately.

Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2015.02.022.

Acknowledgments

This work was supported by the project RePulse- Responses of *Daphnia magna* exposed to chemical pulses and mixtures throughout generations (FCOMP-01-0124-FEDER-019321; Refª. FCT PTDC/AAC-AMB/117178/2010), by funding FEDER through COMPETE- Programa Operacional Factores de Competitividade, and by National funding through FCT-Fundação para a Ciência e Tecnologia. Ana Rita Rego Gouveia Silva was funded by the project "MARPRO-Conservation of marine protected species in Mainland Portugal" through the doctoral fellowship (BD/UI88/5534/2011) and Andreia Cruz was funded by an individual post-doctoral grant (BPD/UI88/2886/2013), within the project "Sustainable Use of Marine Resources" - MARES (CENTRO-07-ST24-FEDER-002033), financed by QREN, Mais Centro- Programa Operacional Regional do Centro e União Europeia/ Fundo Europeu de Desenvolvimento Regional.

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Supplementary data

Table 2.1 SD. Biological interpretation of additional parameters, *a* and *b*, that define the functional from the deviation patterns from the reference models Concentration Addition (CA) and Independent Action (IA). Adapted from Jonker *et al.* 2005.

Deviation ıttern	**Parameter *a* (CA and IA)**	**Parameter *b* (CA)**	**Parameter *b* (IA)**
Synergism/ Antagonism	$a>0$ antagonism $a<0$ synergism		
Dose-ratio dependent	$a>0$ antagonism except for mixture ratios where negative *b* value indicate synergism $a<0$ synergism except for mixture ratios where positive *b* value indicate antagonism	$bi>0$ antagonism where the toxicity of the mixture is caused mainly by toxicant *i* $bi<0$ synergism where the toxicity of the mixture is caused mainly by toxicant *i*	
Dose-level dependent	$a>0$ antagonism at low dose level and synergism at high dose level $a<0$ synergism at low dose level and antagonism at high dose level	$b_{DL}>1$ change at lower EC_{50} level $b_{DL}=1$ change at EC_{50} level $0< b_{DL}<1$ change at higher dose level than the EC_{50} $b_{DL}<0$ no change, but the magnitude is dose level dependent	$b_{DL}>2$ change at lower EC_{50} level $b_{DL}=2$ change at EC_{50} level $1< b_{DL}<2$ change at higher dose level than the EC_{50} $b_{DL}<1$ no change, but the magnitude is dose level dependent

Table 2.2 SD. Summary of physico-chemical properties in *Daphnia magna* reproduction tests upon single exposure to triclosan and carbendazim.

Chemical	**pH**	**Dissolved oxygen concentrations (mg/L)**	**Conductivity (µS/cm)**
Triclosan	7.62 - 8.25	> 6.7	498 - 607
Carbendazim	7.98 - 8.93	> 7.2	493 - 594

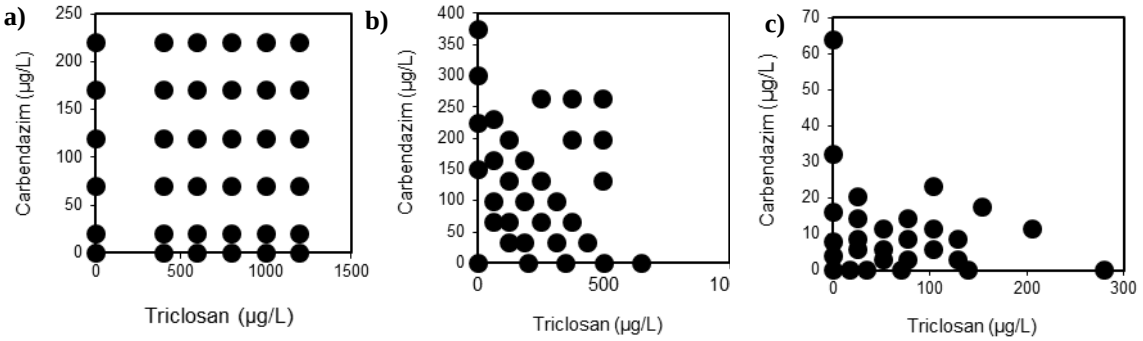


Figure 2.1 SD. A full factorial design used for the immobilisation test (**a**), and fixed ray design for both feeding inhibition test (**b**) and reproduction test (**c**) for the binary mixture exposures of triclosan and carbendazim.

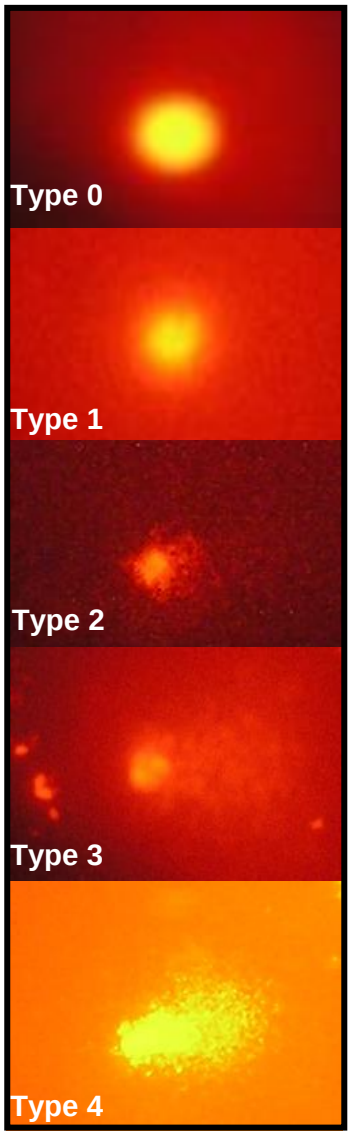


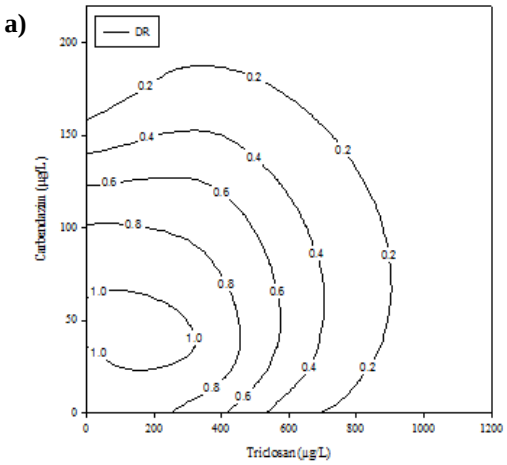
Figure 2.2 SD. Comet type scale used in daphnid cells.

Table 2.3 SD: MIXTOX analysis of the combinations of triclosan and carbendazim to immobilisation, feeding inhibition (24h exposure and 4h post exposure) and reproduction of *D. magna*.

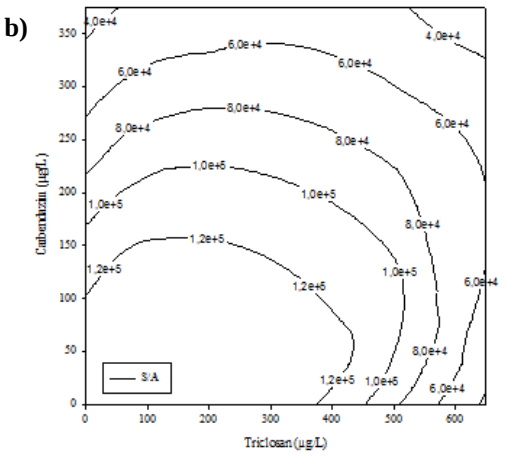
	Endpoint: **Immobilisation** **Concentration Addition**			
	CA	S/A	DR	DL
r^2	0.62	0.75	0.79	0.75
SS	122.57	81.88	67.57	81.04
***p*(*F*-test)**	**9.85×10^{-43}**	-	-	-
$p(\chi^2)$	-	**1.79×10^{-10}**	**0.0015**	0.36
max	0.98	0.98	0.98	0.98
a	-	2.56	1.69	-0.0084
b	-	-	2.05	126.28
	Endpoint: **Feeding inhibition 24h exposure** **Concentration Addition**			
	CA	S/A	DR	DL
r^2	0.24	0.82	0.83	0.82
SS	2.39×10^{10}	5.57×10^{9}	5.31×10^{9}	5.51×10^{9}
***p*(*F*-test)**	0.10	-	-	-
$p(\chi^2)$	-	**8.50×10^{-12}**	0.22	0.58
max	110443.2	133625.9	132317.0	132925.3
a	-	2.80	1.45	4.01
b	-	-	2.55	0.17
	Endpoint: **Feeding inhibition 4h post exposure** **Concentration Addition**			
	CA	S/A	DR	DL
r^2	0.47	0.52	0.52	0.52
SS	4.62×10^{10}	4.26×10^{10}	4.26×10^{10}	4.15×10^{10}
***p*(*F*-test)**	**0.0013**	-	-	-
$p(\chi^2)$	-	0.11	0.22	0.18
max	183141.1	181134.0	182671.0	183515.9
a	-	34.81	228.2	-0.11
b	-	-	-3765.47	15.29
	Endpoint: **Reproduction** **Concentration Addition**			
	CA	S/A	DR	DL
r^2	0.46	0.80	0.81	0.81
SS	16516.76	6021.93	6021.93	6021.85
***p*(*F*-test)**	**0.0045**	-	-	-
$p(\chi^2)$	-	**1.07×10^{-7}**	0.98	0.98
max	176.15	174.07	174.08	174.07
a	-	-0.50	1.12	0.55
b	-	-	-2.01	2.07

r^2 represents the maximum likelihood test; SS represents the sum of squared residuals; p(*F*-test) represents the result of the likelihood ratio test; $p(\chi^2)$ represents the outcome of the likelihood ratio test; max represents the control response, *a* and *b* represents the additional parameters of the function; CA represents the concentration addition model; S/A represents synergism/antagonism; DR represents the dose ratio dependence; DL represents the dose level dependence.

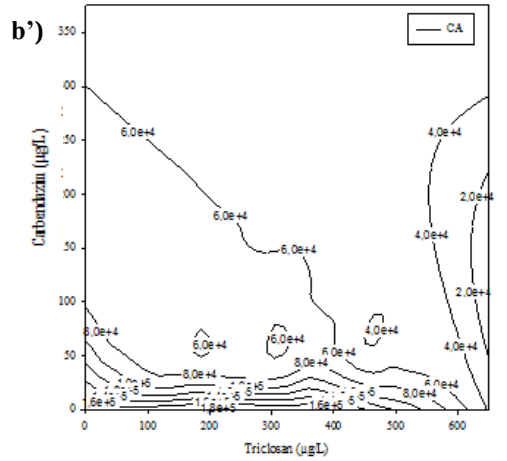
a)


DR
Carbendazim (µg/L)
Triclosan (µg/L)

b)


S/A
Carbendazim (µg/L)
Triclosan (µg/L)

b')


CA
Carbendazim (µg/L)
Triclosan (µg/L)

c)

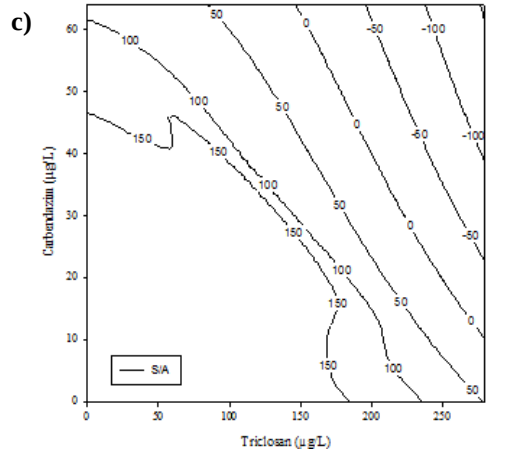

S/A
Carbendazim (µg/L)
Triclosan (µg/L)

Figure 2.3 SD. Concentration-response relationship for the binary mixture of triclosan and carbendazim (2D isobolic surfaces). a) Survival of *Daphnia magna* (Pi) (n=5, 3 replicates showing a DR deviation from the CA model b) Feeding rate of *D. magna* (cells/mL/ind/hr) at 24h (n=5, 1 replicate) showing antagonism from the CA model b') Feeding rate of *D. magna* (cells/mL/ind/hr) at 4h post-exposure (n=5, 1 replicate) showing no deviation to the CA model c) Reproduction of *D. magna* (number of total neonates per female after 21 days of exposure) (n=1, 1 replicate) showing synergistic pattern to the CA model.

Table 2.4 SD: MIXTOX analysis of the combinations of triclosan and carbendazim to % of no DNA damage on *D. magna*.

	Endpoint: % of no DNA damage Concentration Addition			
	CA	S/A	DR	DL
r^2	0.88	0.88	0.92	0.89
SS	728.34	727.31	516.70	693.58
***p*(*F*-test)**	$1.62x10^{-14}$	-	-	-
$p(\chi^2)$	-	0.82	**0.0017**	0.40
max	72.68	72.77	71.34	72.61
a	-	-0.12	-4.02	-1.99
b	-	-	12.36	0.87

r^2 represents the maximum likelihood test; SS represents the sum of squared residuals; *p*(*F*-test) represents the result of the likelihood ratio test; $p(\chi^2)$ represents the outcome of the likelihood ratio test; max represents the control response, *a* and *b* represents the additional parameters of the function; CA represents the concentration addition model; S/A represents synergism/antagonism; DR represents the dose ratio dependence; DL represents the dose level dependence.

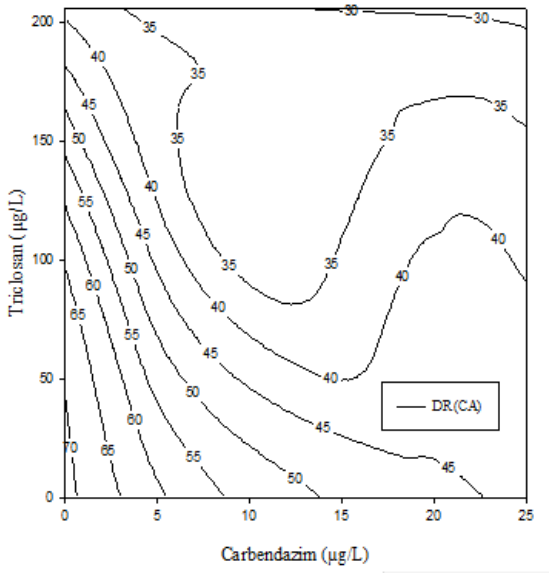


Figure 2.4 SD. Concentration-response relationship for the binary mixture of triclosan and carbendazim (2D isobolic surfaces) for the % of no DNA damage on *D. magna* cells showing a DR deviation from the CA model.

Chapter 3

Multigenerational effects of carbendazim in Daphnia magna

Published by Environmental Toxicology and Chemistry

doi: 10.1002/etc.3541

Multigenerational effects of carbendazim in *Daphnia magna*

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Abstract

Carbendazim is a fungicide largely used in agriculture as a plant protection product. Due to agricultural runoffs, drainage and/or leaching it reaches surface waters at concentrations possibly hazardous to aquatic communities. Due to potential and continuous release of carbendazim to aquatic systems, long term exposure to aquatic organisms should be addressed. To fill the knowledge gap on this, the present study aimed at evaluating the responses of multigenerations of *Daphnia magna* (clone K6) to an environmental relevant concentration of carbendazim (5 µg/L); 12 successive generations were evaluated and the effects in these offsprings were compared to those from a control population. Neonates' fitness was assessed through immobilisation, reproduction and feeding activity tests along with the comet assay for the *in vivo* DNA damage evaluation. In addition, recovery from long-term exposure was also assessed. In the F5 generation, the results indicated that when daphnids were re-exposed to carbendazim, DNA damage was higher in daphnids continuously exposed to carbendazim than those from clean medium. After daphnids were moved to clean medium, a low recovery potential was observed for DNA damage. Daphnids exposed continuously for six generations (F6) to carbendazim presented an increase on feeding rates when re-exposed to carbendazim compared with F6 daphnids reared in clean medium. The continuous exposure to carbendazim induced a significant increase in DNA damage from F0 to F12 generation. Deleterious effects of the multigenerational exposure to carbendazim were more notorious at a subcellular level (DNA damage) compared with the individual level.

Key words: *Daphnia magna*, multigenerational effects, toxicity, carbendazim, genotoxicity

1. Introduction

The increasing use of pesticides has promoted the contamination of surface waters, mainly due to runoffs, drainage and/or leaching inducing deleterious effects in organisms and populations and therefore jeopardizing ecosystems (Daam and Brink, 2007; Ongley, 1996). Carbendazim (CBZ) (methyl-2-benzimidazole carbamate) is widely used in agricultural practices as a plant protection product (WHO, 1993) and has already been detected in Thailand, Spain (Guadalquivir river basin) and Chile (Traiguén river basin) at concentrations that can reach 4.5 µg/L in surface waters (Chatupote and Panapitukkul, 2005; Masia *et al.*, 2013; Palma *et al.*, 2004). CBZ is authorized at a national level in some countries, it is applied in many different crops (EU Pesticide Database, 2015), at different times (*e.g.* from spring to autumn) being persistent in the water column (half-life of 6 to 25 weeks) (Cuppen *et al.*, 2000). Therefore in the environment, aquatic non-target organisms might be long-term exposed to this pesticide and studies evaluating long-term effects at ecological relevant concentrations of chemical compounds are essential given the continuous release of pesticides to aquatic environments.

Carbendazim has been shown to negatively affect freshwater zooplankton species, reflected by an increase in the immobilisation and reduced feeding activity (Ferreira *et al.*, 2008; Ribeiro *et al.*, 2011). In addition, it can potentially act in the mitosis during daphnids embryonic development leading to aborted eggs, potentially affecting the viability of juveniles (Canton, 1976; Ribeiro *et al.*, 2011; Chapter 2 - Silva *et al.*, 2015). However, to our knowledge, long-term multigenerational effects of CBZ in invertebrates and in daphnids in particular have never been assessed. Generally, parental exposure can alter offspring sensitivity and offsprings may become more sensitive or less sensitive (induced tolerance). The increase in tolerance throughout generations might be due to physiological changes (acclimation) or can have a genetic basis (adaptation) (Bodar *et al.*, 1990; LeBlanc, 1982).

Cladocera like *Daphnia* sp. have features that make them ideal test-species to be used in long-term experiments. Multigenerational tests using parthenogenetic daphnids raised in laboratory conditions eliminates genetic variability (Hebert and Ward, 1972). Due to the absence of recombination, asexually-reproducing species are more vulnerable to mutations and DNA damage throughout generations (Simon *et al.*, 2003; Sukumaran and Grant, 2013). *Daphnia magna* has been used in standard toxicity tests and reproduction

tests are used for regulatory purposes due to their high level of sensitivity (OECD, 2008). Evaluating neonates' fitness by assessing survival, feeding or reproduction upon parental exposure should thus be evaluated, increasing the cost-effectiveness and the profit of these tests (Sánchez *et al.*, 2000; van Leeuwen *et al.*, 1985).

Although multigenerational studies with *D. magna* have been conducted with pesticides, the majority have only assessed effects throughout a reduced number (two or three) of generations (Brausch and Salice, 2011; Chen *et al.*, 2013). Results with two generations might lead to different findings regarding tolerance patterns, compared with a study with more generations, because tolerance might appear only after a certain number of generations, changes in tolerance might occur throughout the exposed generations, and may be cumulative. Thus different assumptions regarding organisms' sensitivity might be drawn (Postma and Davids 1995). Therefore, looking at longer exposure periods and long-term effects throughout a higher number of generations will provide a more accurate insight on the toxicity of xenobiotics in individuals but also transposing to the population level.

In the present study, we aimed at understanding the long-term effects of continuous exposures to CBZ in the cladocera *D. magna.* For that, daphnids were exposed to an environmental relevant concentration of CBZ (5 µg/L) throughout twelve generations. Immobilisation, feeding inhibition and/or reproduction assays were carried out using neonates originated from exposed and unexposed mothers in selected generations (Fig. 3.1). Since DNA damage was already detected in D. magna exposed to CBZ in previous works (Chapter 2 – Silva *el al.* 2015), the comet assay was used as a complementary test to detect the occurrence of DNA damage as an early warning indicator of CBZ genotoxic effects (Sukumaran and Grant, 2013).

In real scenarios, environmental conditions might change and it is also important to study whether organisms are able to recover from a chemical stress episode and in turn determine if they develop chemical tolerance (Massarin *et al.* 2010). This was taken into consideration in the present study by evaluating the DNA damage in neonates born from F5, after being kept for 13 days in clean medium.

2. Materials and methods

2.1 Test organism

D. magna Straus clone K6 (originally from Antwerp, Belgium) was obtained from well-established laboratory cultures (Department of Biology, University of Aveiro, Portugal), kept in American Society for Testing and Materials (ASTM) moderated-hard-water medium (ASTM, 1980), at controlled conditions of temperature (20ºC±1ºC) and photoperiod (16h light-8h dark). *D. magna* was cultured in 1L glass vessels containing culture medium and 25 daphnids. The medium was renewed three times a week and daphnids were fed with *Raphidocelis subcapitata* (formerly known as *Pseudokirchneriella subcapitata*) at a concentration of $3x10^5$ cells/mL and supplemented with an organic extract (Marinure seaweed extract, supplied by Glenside Organics Ltd.).

2.2 Test chemicals

CBZ with a chemical purity of 99.4% was obtained from Bayer Crop Science. A stock solution of CBZ was prepared in ASTM medium to maintain the multigenerational cultures, with no solvent as the low concentration used was capable of being dissolved without any carrier. For the immobilisation, feeding inhibition and reproduction tests, a stock solution of CBZ was prepared in ASTM and acetone as solvent due to the low solubility of CBZ at higher concentrations. A solvent control of 100 µL acetone/L was also included in all experimental setups as recommended by the OECD guideline 23 (OECD, 2000).

CBZ concentrations in the test medium were determined by chemical analysis performed every 12 hours. For that, an extra beaker was used with ASTM medium simultaneously contaminated with those for exposure testing. Samples were analysed by the Marchwood Scientific Services (Southampton, UK) through Liquid Chromatography-Mass Spectrometry (LCMS-MS) using the Querchers method. A representative portion of the sample (200-300 mL) was extracted with 20 mL of acetonitrile (containing 1% acetic acid). This was followed by a partitioning step with magnesium sulphate and a subsequent buffering step with sodium acetate. After mixing an aliquot with methanol, the extract was injected directly into the LC-MS/MS system (instrument Agilent 6410 Triple Quad LCMs-MS) without any clean-up. A 10 µl injection volume was utilized. Standards were prepared

in solvents at seven levels with recoveries in the range 70-120%. Chemical analysis data were then included in a decay model, to assess the degradation constant in time:

(Equation 1) $C_t = C_0 e^{-k0t}$

Where C_0 corresponds to the initial external concentration (µg/L), K_0 corresponds to the constant of degradation of the chemical in the medium (/h) and *t* corresponds to time (h) (Widianarko and Van Straalen, 1996).

2.3 Multigenerational experimental setup

Two isoclonal populations of *D. magna* were used in the multigenerational experimental setup: *1)* one population was exposed continuously to 5 µg/L of CBZ (Dph_CBZ) and *2)* another population (Dph_Clean) was kept under similar culture conditions (in ASTM water, fed *R. subcapitata* supplemented with organic extract but with no CBZ). The present experiment lasted for approximately 34 weeks, corresponding to twelve generations. This CBZ concentration (5 µg/L) corresponds to the no observed effect concentration (NOEC) value determined for *D. magna* reproduction in Chapter 2 - Silva *et al.* 2015.

Both clean (Dph_Clean) and contaminated (Dph_CBZ) isoclonal populations of *D. magna* were reared in similar conditions in 1 L glass vessel containing 25 daphnids, with medium renewal three times a week and daphnids fed with *R. subcapitata* at a concentration of $3x10^5$ cells/mL and supplemented with an organic extract (Marinure seaweed extract, supplied by Glenside Organics Ltd.). Populations were monitored every day and the first and second brood were discarded. *D. magna* neonates from the third to fifth brood (< 24h old) were used to start new generation trials, and the same brood was used for both Dph_Clean and Dph_CBZ. The experimental setup included only one vessel/replicate per population as we aimed at increasing efforts on the generations' number and neonates' fitness testing (see below) in detriment of replicates. In addition, as daphnids are clonal organisms the variability within replicates due to genetics may be lower than for other organisms.

Neonates used for the ecotoxicity tests spent <24h in the same environment as the mothers. Throughout the multigenerational experiment, the two isoclonal populations (Dph_Clean and Dph_CBZ) were synchronised in their reproduction, with a difference of some hours.

2.3.1 Comet assay

The comet assay was performed in neonates of *D. magna* at F0, F6, F9 and F12 in both Dph_Clean and Dph_CBZ (Fig. 3.1) in order to detect DNA damage and relate it to daphnids fitness throughout generations. Four replicates were used for each isoclonal population (Dph_Clean and Dph_CBZ), each replicate consisted of a pool of fifteen neonates (<48h).

In addition, to evaluate the potential recovery of *D. magna* after a multigenerational exposure to CBZ, F5 neonates from Dph_CBZ were transferred to clean medium for 13 days and till their 3rd brood was released. These neonates were pooled and also used in the comet assay as described above (four replicates of fifteen neonates each).

The comet assay methodology employed was the same as described by Nogueira *et al.* (2006) and Silva *et al.* 2015 – Chapter 2. For all cases, organisms were immediately processed for the comet assay and four replicates with fifteen juveniles of *Daphnia* (< 48h) each were used. Positive controls consisted on daphnid's cells exposed to hydrogen peroxide (H_2O_2). To prevent UV induced DNA damage, the assay was conducted under yellow light. Briefly, organisms were placed in 1.5 mL *Eppendorfs* containing 1 mL of phosphate-buffered saline (PBS), 10% dimethyl sulfoxide (DMSO) and 20 µM ethylenediamine tetra-acetic acid (EDTA). Then, organisms were gently disintegrated mechanically and samples were centrifuged (200 *g*) for 10 min at 4ºC and most of the supernatant was gently removed. From the pellet (containing cells), 10 µL were transferred to *Eppendorfs* containing 0.5% low melting point agarose (at 37ºC). The mixture was spread on the microscope glass slides containing 1% normal melting agarose, and coverslips were applied. Slides were placed on ice for 10 min. Following that, slides were placed in a solution of 10 mM Tris-HCl, 100 mM EDTA, 2.5 M NaCl, 10% DMSO, 10% Triton X-100, pH 10, for cell lysis and allocated in the electrophoresis tin, which already contained an electrophoresis solution, for 15 min before allowing DNA denaturation and unwinding. An electric current of 300 mA was then applied for 10 min. For neutralization, slides were washed with 0.4 M Tris-HCl (pH=7.5) and then dehydrated with absolute ethanol 100% for 10 sec; finally the slides were left to dry for 1 day in the dark.

For image analysis, slides were stained with 100 µL ethidium bromide (20 µL/mL), overlaid with a coverslip and observed in a fluorescence microscope (Olympus BX41TF, China) at 400x magnification. One hundred cells per slide were randomly selected and

examined. The scorer was unaware of the treatment condition when reading the slides. DNA damage was visually scored: each cell was scored on a 0 to 4 scale as described by Duthie and Collins (1997). Type 0 represents no DNA damage, type 1 and 2 represent mild to moderate damage, respectively and type 3 and 4 represent extensive DNA damage. The total comet score was calculated according to the method of Duthie and Collins (1997): (number of cells in type 0 × (type) 0) + (number of cells in type 1 × (type) 1) + (number of cells in type 2 × (type) 2) + (number of cells in type 3 × (type) 3) + (number of cells in type 4 × (type) 4). Therefore, the total score for 100 cells could range from 0 (all comets with no damage) to 400 (all comets with maximum damage). A percentage of DNA damage was then calculated. Fig. 3.1 SD represents a comet type scale in daphnid cells.

2.4 Ecotoxicity tests

Throughout the experimental procedures/generations, neonates' fitness was evaluated by testing their sensitivity to CBZ. The experimental design is represented in Fig. 3.1. Tests using organisms from both Dph_Clean and Dph_CBZ were performed simultaneously in order to control differences in daphnids' responses due to sensitivity variations within organisms (Loureiro *et al.*, 2010). In all ecotoxicity tests, an acetone control was run simultaneously with the negative control and CBZ treatments.

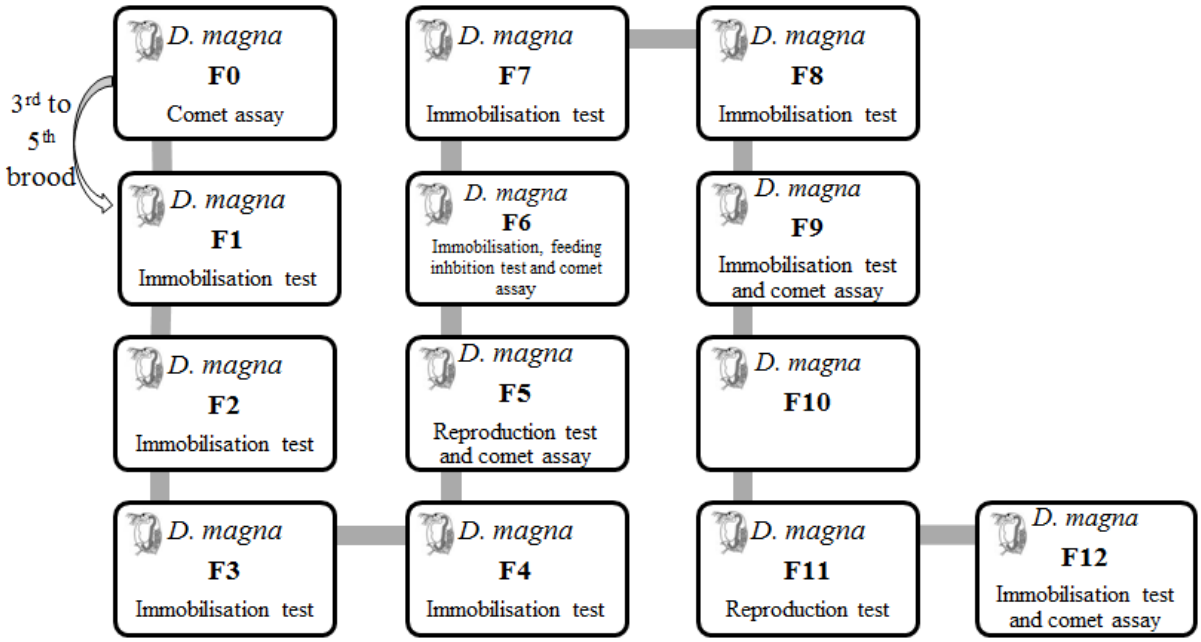

Figure 3.1. Experimental design of the multigenerational test with *Daphnia magna* to assess long-term effects due to carbendazim exposure. Each box represents a generation and the respective tests carried out.

2.4.1 Immobilisation tests

Acute toxicity tests were adapted from the OECD guideline 202 (OECD, 2004) and were carried out for almost all generations except F5, F10 and F11 (Fig. 3.1). Neonates with less than 24h were exposed in 50 mL glass beakers to CBZ concentrations ranging from 25 to 200 µg/L, without food (16:8h light:dark photoperiod and 20±1ºC). After 24h and 48h of exposure, immobilised neonates were recorded. The experimental setup consisted of three replicates of five neonates each, for every CBZ and control experimental treatments.

2.4.2 Feeding inhibition test and post-exposure tests

Feeding inhibition bioassays were carried out in accordance to the methodology described by McWilliam and Baird (2002). As no clear changes in sensitivity were observed between the Dph_Clean and Dph_CBZ until the F4 generation for the immobilisation endpoint (data shown later), the F6 generation was chosen to perform the feeding inhibition test. This approach aimed at evaluating a more sensitive endpoint which could possibly provide some hints on sensitivity changes between isoclonal populations (exposed and non-exposed). F6 neonates (<24 h) Dph_Clean and Dph_CBZ were moved to a new culture aquarium (4L) and maintained in the same exposure conditions until reaching the fourth instar (5-6 days old). This life stage enables to run feeding trials within a single moult interval, avoiding moulting interference on feeding activity. CBZ concentrations ranged from 50 to 450 µg/L. Three replicates per experimental treatment and controls with five organisms each were used. Each replicate consisted of 170 mL glass beakers containing 100 mL ASTM, *R. subcapitata* at a concentration of $5x10^5$ cells/mL (with CBZ or under controls' conditions). Daphnids were allowed to feed for 24h at 20ºC, under darkness. To control for algae growth during the test, a blank set of 50 mL beaker with one replicate was also prepared for each treatment and controls, at the same conditions as described previously, but without daphnids. Following the exposure period, daphnids from each replicate were transferred into 50 mL beakers with clean ASTM and *R. subcapitata* (cell density of $5x10^5$ cells/mL) for 4h in the dark (post-exposure period) in order to assess post-exposure effects. In the post-exposure period, five blanks were also included. Feeding rates (cells/mL/individual/h) were determined in accordance to Allen *et*

al. (1995); algal concentration was measured using a colorimetric method at 440 nm for both exposure and post-exposure periods (Pérez *et al.*, 2011).

2.4.3 Reproduction tests

The reproduction test with F5 and F11 neonates (Fig. 3.1) followed the OECD guideline 211 (OECD, 2008), with few adaptations. The experimental setup included five replicates per treatments and controls with one neonate each (< 24h). Each replicate consisted of 50 mL glass beakers with ASTM medium, *R. subcapitata* (cell density of $3x10^5$ cells/mL) and an organic extract (16:8h light:dark photoperiod and 20±1ºC). Test solutions were renewed every other day and daphnids fed daily. For 21 days, the survival, the number of neonates, the time for the first brood and the number of broods were recorded. Aborted eggs and abnormal characteristics were registered as well. Adult's body length (in mm, excluding the anal spine) after the 21 days of exposure was determined under a stereomicroscope (MS5, Leica Microsystems, Houston, USA). Physico-chemical parameters: pH, electrical conductivity and dissolved oxygen were recorded at the beginning, middle and end of the tests. Four CBZ concentrations ranging from 5 to 50 µg/L were tested.

Data collected from generations F5 and F11 reproduction tests, were used to calculate the intrinsic rate of natural increase (*r*) using the Euler Lotka equation (Lokta, 1913):

(Equation 2) $\sum_{x=0}^{x} lx\ mx\ e^{-rx} = 1$

where *lx* is the proportion of individuals surviving to age *x*, *mx* is per-capita fecundity, and *x* is days.

To compare responses of both populations from CBZ contaminated (Dph_CBZ) and clean medium (Dph_Clean), neonates from the 3rd brood obtained in the F5 reproduction test (controls, 5 and 20 µg/L) were also used to detect DNA damage (four replicates of fifteen neonates each).

2.5 Statistical Analysis

Data normality was assessed using residual probability plots for all variables (Minitab Version 14.0, 2003). Homoscedascity of data was assessed using Levene's equal variance test. Within the multigenerational exposure, for the DNA damage evaluated in F0, F6 F9 and F12, significant effects of the generation number and populations (fixed factors) were checked using a two-way ANOVA with Bonferroni post-test (Systat Software Inc., 2008). To evaluate the percentage of variance accounted for each factor in the ANOVAs, the R-squared was calculated by dividing the sum of squares of each factor and of their interaction by the total sums of squares of the two-way ANOVAs (Hullett and Levine, 2003).

For the immobilisation, feeding inhibition and reproduction assays differences between the negative control and the solvent control were checked using a t-test. To detect significant differences between populations (Dph_Clean and Dph_CBZ), regarding their reproduction, feeding activity and DNA damage in the ecotoxicity tests, a two-way ANOVA was performed using SigmaPlot (Systat Software Inc., 2008), with CBZ concentrations and populations as fixed factors.

48h-LC_{50} values (immobilisation) were estimated using probit analysis (Minitab Version 14.0, 2003). The mean value of the intrinsic rate of natural increase (*r*) was determined using the Jackknife method (Pestana *et al.*, 2010; Taberner *et al.*, 1993). The EC_{50} values were calculated using a nonlinear regression with a sigmoid or logistic function, while consistently choosing the best fit using SigmaPlot (Systat Software Inc., 2008). LC_{50} and EC_{50} values were statistically compared according to Sprague and Fogels (Sprague and Fogels, 1976).

3. Results and Discussion

3.1 Chemical analysis

Carbendazim concentration in the ASTM decreased over time, with a decay rate (K_0) of 0.03/h (SE=0.005), showing that only 18% of the initial concentration (7.2 µg/L) remained after 48h (as also described in Chapter 2 - Silva *et al.* 2015). Although this is a significant decay of the compound, medium was changed three times a week (in the

Dph_Clean and Dph_CBZ populations) and every other day (in the reproduction tests), and therefore nominal concentrations were used for the LC/EC_{50} calculations, as advised by the OECD 211 guideline (Annex 6). The chemical behaviour observed is somehow contradictory to what has been described by Cuppen *et al.* (2000), which found that carbendazim was persistent, with a half-life of 6 to 25 weeks in the water column.

3.2 Multigenerational effects

The experimental setup in the multigenerational experiment included no replication. However, as daphnids reproduce via cyclical parthenogenesis, they are clonal organisms. The offspring is genotypically identical to their mother and therefore the variability within replicates due to genetics might be lower than for other species (Hebert and Ward, 1972). Replication was then included for the toxicity tests as described below. As a global perspective of the work performed, daphnids did not show a clear pattern towards tolerance or resistance. Resistance on the parthenogenic *D. magna* might be related to the presence of resistant genotypes in some populations, which are only expressed when needed (Brausch and Smith, 2009). Lopes et al. (2006) demonstrated the presence of genetically determined resistance in field populations of *Daphnia longispina* due to long term exposures to acid mine drainage. Gustafsson et al. (2005) found that *D. magna* developed tolerance to toxic *Mycrocystis* in two generations. This tolerance was an inducible defence mechanism and this trait can be transferred from mothers to progeny (Gustafsson et al., 2005). On the other side, other authors have also argued that the development of resistance to some toxic compounds in aquatic organisms in a long-term exposure might be difficult to achieve. This may be explained by the need for high fitness costs: high energy acquisition and consumption (Dietrich et al., 2010; Muller et al., 2010).

In the present study, a relationship in sensitivity between the lethal and sublethal endpoints throughout generations was not found. Several authors have reported the existence of no (genotype) concordance between lethal and sublethal responses to different chemicals (Barata et al., 2000; Lopes et al., 2005). The authors stated that specific mechanisms rule the lethal responses, while general mechanisms rule the sublethal responses (Baird et al., 1990; Lopes et al., 2005).

The comet assay was applied to test genotoxic effects caused by CBZ on *D. magna* throughout generations. DNA strand breaks have been considered a more sensitive

endpoint than reproduction and growth (Atienzar et al., 2001). Therefore, detection of DNA damages should be assessed whenever possible as a complement to other physiological parameters. After six generations (F6), DNA damage was significantly higher in Dph_CBZ population when compared to Dph_Clean population (Fig. 3.2). Corroborating this result, the same occurred in the 9th and 12th generation (F9 and F12) where daphnids from Dph_CBZ showed a significant increase in the percentage of DNA damage in comparison to Dph_Clean daphnids (Fig. 3.2).

In the Dph_CBZ population the DNA damages increased throughout the generations (from F0 to F12) with the highest percentage of DNA damage observed in the F12 daphnids, suggesting a multigenerational effect of CBZ (Fig. 2). DNA damage differed between the two populations (two-way ANOVA, $F_{1,28}$ = 261.0, p<0.001) and it differed between the generations as well (two-way ANOVA, $F_{3,28}$ = 21.0, p<0.001) (Table 3.1), being clearly higher in Dph_CBZ. Although the population factor explained the majority of the total variation (76%), a significant interaction between the two factors was also evident (two-way ANOVA, $F_{3,28}$ = 5.3, p<0.05) (Table 3.1), meaning that the populations reacted differently throughout the generations for this endpoint.

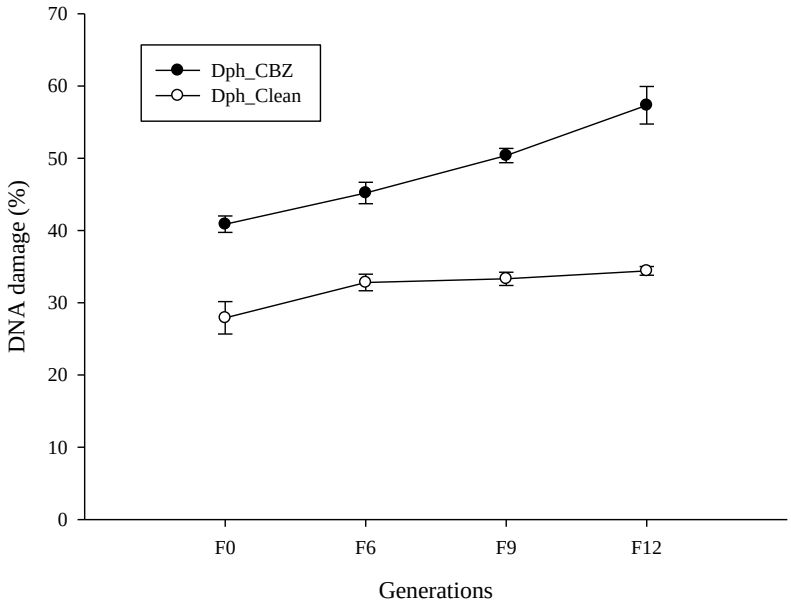

Figure 3.2. DNA damage (%) in cells of *Daphnia magna* from F0, F6, F9 and F12 generations for both populations Dph_Clean (white dots) and Dph_CBZ (black dots). Data are expressed as mean values and standard error.

Table 3.1. Two-way ANOVA results testing for effects of populations of *Daphnia magna* (Dph_Clean *vs.* Dph_CBZ), generations and their interaction on the percentage of DNA damage.

	DF	Sum of squares	F	*p*-value	R^2
DNA damage (%)					
Generations	3	457.2	21.0	**<0.001**	0.18
Population	1	1895.9	261.0	**<0.001**	0.76
Generations x Population	3	116.2	5.3	**0.007**	0.05

The exposure of *D. magna* mothers to an environmental relevant concentration of CBZ caused damage at the DNA level and these damages were possibly cumulative and passed throughout the generations affecting the overall performance of the offspring. Corroborating this hypothesis, Atienzar and Jha (2004) demonstrated a transmission of DNA damage from parents to neonates after exposure to benzo(a)pyrene. Plaire et al. (2013) evaluated the DNA damage, using the Random Amplified Polymorphic DNA technique, and observed that daphnids exposed to depleted uranium accumulated DNA damage and it was transmitted to offspring along with an increase in the toxic effects. Another study has demonstrated transmission and accumulation of DNA alterations throughout generations of *D. magna* exposed to gamma radiation (Parisot et al., 2015). Additionally, rather than the occurrence of DNA damage accumulation/transmission throughout generations, genotoxicity (DNA damage) is dependent upon the induction and efficiency of several repair mechanisms as well and these mechanisms might fail throughout the generations (Jha, 2008). Both DNA damage and abnormal mitosis are promoters of genomic instability, and a relationship between both has already been reported (Ganem and Pellman, 2012) possibly causing a decrease in populations fitness and consequently with costs for future generations.

The results presented here show that the comet assay was a sensitive method and should be used as a complementary test to other standardized ecotoxicological tests.

Related to these effects at the DNA level, CBZ is known to induce the abortion of eggs, due to a possible effect on egg mitosis, similar to CBZ mode of action in fungi (Canton, 1976; Ribeiro et al., 2010), and also to induced DNA damage in daphnids (Silva et al., 2015). Daphnids ability to recover from DNA damage upon exposure to different contaminants has been observed in some works (Atienzar and Jha, 2004; Plaire et al., 2013), as an indication of their degree of repair capacity when returned to favourable

conditions (Atienzar and Jha, 2004; Plaire et al., 2013). Regarding daphnids recovery, Guan and Wang (2006) observed that *D. magna* recovered from cadmium exposure after one generation only. In the present work, DNA damage recovery did not seem to occur, since no decrease on DNA damage was observed upon a 13-day exposure of daphnids to clean medium. Here, offsprings born from Dph_CBZ presented a higher percentage of DNA damage than daphnids from Dph_Clean population (Fig. 3.6, more details described later). Therefore, these results highlight that DNA damage was apparently cumulative and transmitted throughout generations and was maintained after return to favourable conditions (clean medium). Theodorakis et al. (1998) observed a relation between DNA damage and reproduction endpoints (fecundity and embryo abnormalities) when *Gambusia affinis* was exposed to different radionuclides. Despite this direct relationship between DNA damage and reproduction was not observed in our study, DNA damage might be an earlier indicator of upcoming effects at the population level, as observed in the study of Plaire et al. (2013).

3.3 Ecotoxicity tests

In the experimental setup, proposed neonates were exposed to several concentrations of CBZ and immobilisation, feeding activity and reproduction output were evaluated. Throughout this process, no significant differences were observed between the solvent control used and the negative control (t-test, $p>0.05$). All effects were attained by comparing responses in chemical treatments to those from the solvent control group.

3.3.1 Immobilisation tests

Concerning acute exposures, a slight variability between 48h-LC_{50} values throughout generations was observed even in Dph_Clean. Variability in 48h-EC_{50} values between control populations was also witnessed in the work of Ward and Robinson (2005) when *D. magna* was exposed to cadmium throughout eight generations. To overcome these sensitivity variations within daphnids' responses, tests were performed at the same time with Dph_Clean and Dph_CBZ for each generation, as previously stated. Throughout generations, the Dph_CBZ population did not follow a clear pattern of increase or decrease in sensitivity (Fig. 3.3). Sensitivity between both Dph_Clean and Dph_CBZ populations,

compared in each respective generation, was similar for almost all generations except in F3, F8 and F9 where the 48h-LC_{50} values were significantly different ($p<0.05$) (Fig. 3.3). In these generations, Dph_CBZ appeared to be less sensitive to CBZ, which was indicated by the increase in the 48h-LC_{50} values. However, in the following generations, 48h-LC_{50} values returned to being similar, between Dph_Clean and Dph_CBZ. Different patterns for increasing, or decreasing sensitivity have also been reported in literature by studies comparing organisms exposed to clean *vs.* contaminated medium. Stoddard and Harper (2007) evaluated the effects of copper on four generations of *D. magna*, and observed a non-significant trend towards tolerance, while an increase in acute tolerance was also observed in *D. magna* exposed to cadmium over three successive generations (Bodar et al., 1990). The results from Cd exposure suggested that the tolerance to this metal was not hereditary and was possibly a physiological adaptation (Bodar et al., 1990). In another study, a two-generations exposure to pentachlorophenol caused an increased mortality in *D. magna* (Chen et al., 2013).

The different patterns of sensitivity within multigenerational studies can be related both to the chemical compound used but also to the number of generations tested. In the present study, results regarding the lowest generations (e.g. third generation), provided different conclusions regarding organisms' sensitivity. The variability observed in the LC50 values in our study emphasized the validity of using more than two/three generations and the need of cautious interpretation of data showing small variations in sensitivity in a limited number of generations.

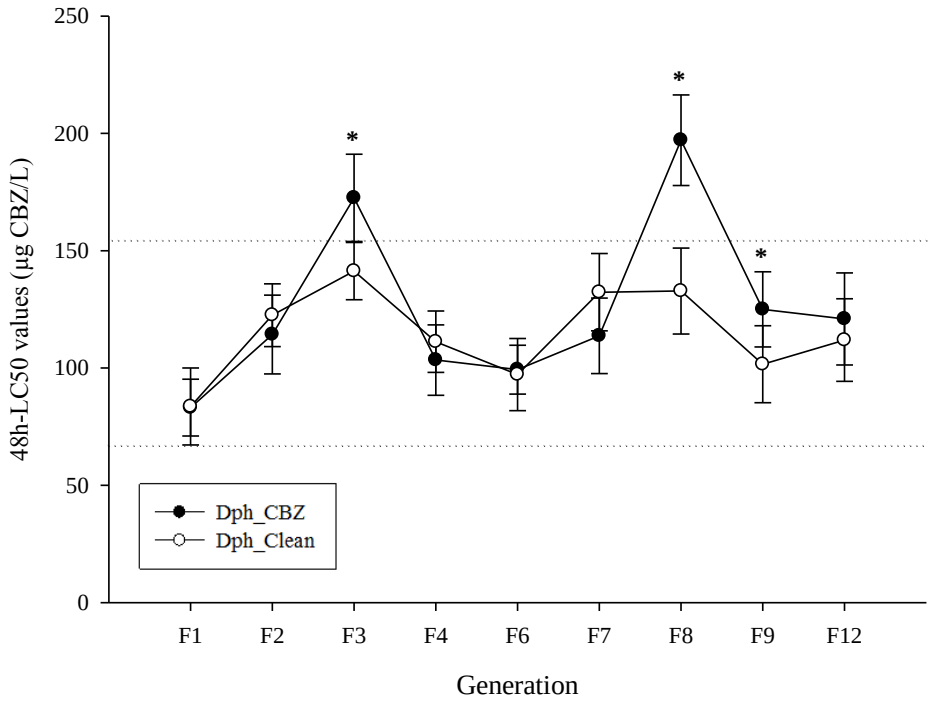

Figure 3.3. Change in the sensitivity to carbendazim (as immobilisation data) expressed as the 48h-LC_{50} with confidence intervals (95%) throughout successive generations of *Daphnia magna* neonates originating from Dph_Clean (white dots) and Dph_CBZ (black dots) populations. Dashed lines represent visually the highest 95% confidence limit (upper line) and the 95% lowest confidence limit of all the LC_{50} values calculated for Dph_Clean (*stands for significant differences between Dph_Clean vs Dph_CBZ LC_{50} values in each generation, $p<0.05$).

3.3.2 Reproduction tests

Daphnids reproductive output, upon exposure to a range of CBZ concentrations, was evaluated in F5 and F11 generations from both populations (Dph_CBZ and Dph_Clean). CBZ exposure reduced the number of produced neonates in F5 Dph_Clean population (EC_{50}= 25.5 µg/L, SE 1.3) and in F5 Dph_CBZ population (EC_{50}= 21.9 µg/L, SE 1.7) in a similar way ($p>0.05$) (Fig. 3.4a).

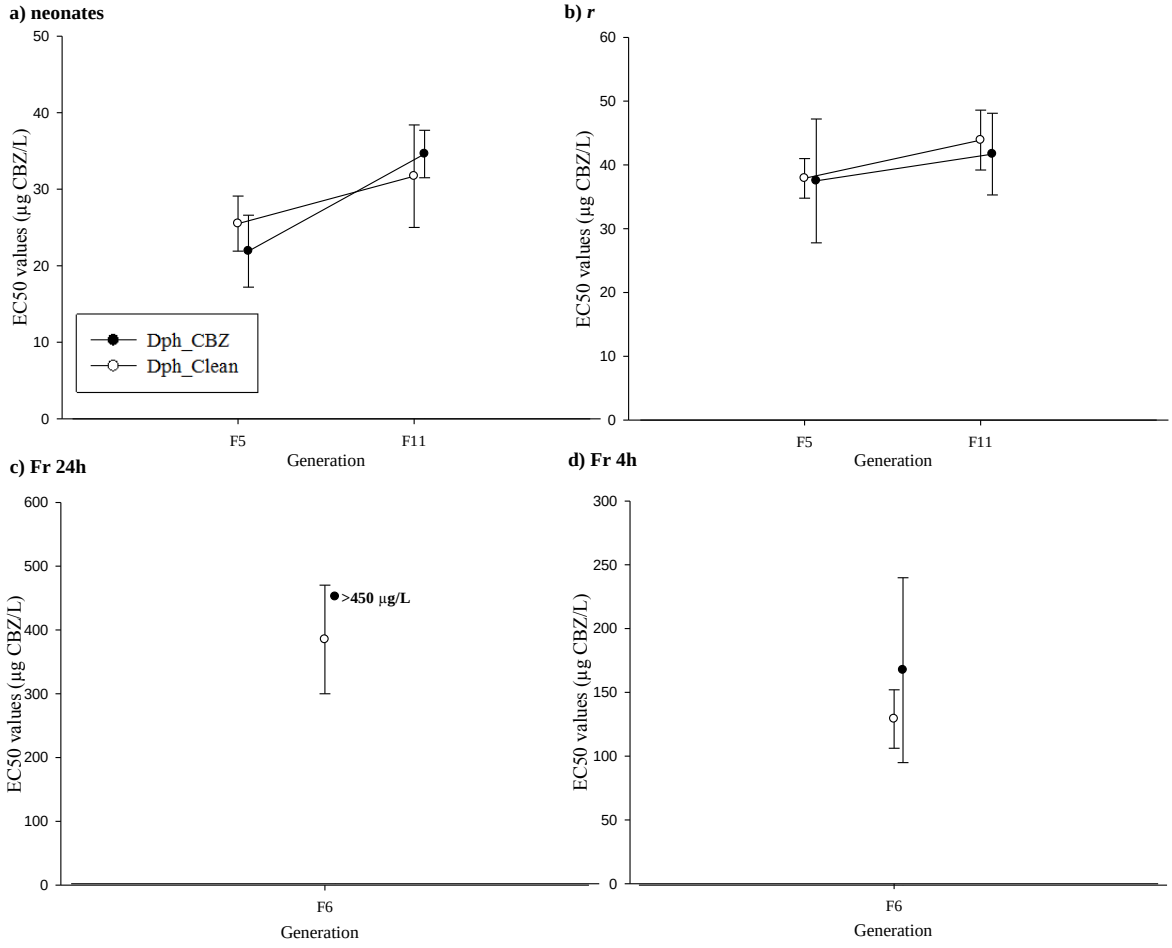

Figure 3.4. EC_{50} values (µg/L) with confidence intervals calculated for the exposure to carbendazim for *Daphnia magna* populations: Dph_Clean (white dots) and Dph_CBZ (black dots), for several endpoints: **a)** number of neonates **b)** *r* = Intrinsic rate of natural increase **c)** Fr= Feeding rate 24h **d)** Fr= Feeding rate 4h post-exposure.

Significant effects of CBZ were detected for the number of neonates (two-way ANOVA, $F_{4,45} = 108.2$, $p<0.001$) and for the number of aborted eggs (two-way ANOVA, $F_{4,45} = 85.3$, $p<0.001$) (Fig. 3.5a and 3.5b and Table 3.2). Comparing the number of neonates, in the F5 reproduction test between Dph_Clean and Dph_CBZ populations, no significant differences were observed (two-way ANOVA, $F_{1,45} = 0.005$, $p>0.05$) (Fig. 3.5a and Table 3.2). No interaction between both factors was found, indicating that populations responded similarly to the concentrations of CBZ (two-way ANOVA, $F_{4,45} = 1.0$, $p>0.05$). However, considering the number of aborted eggs, significant differences were observed between populations (two-way ANOVA, $F_{1, 45} = 7.0$, $p<0.05$). Nevertheless, the population

factor explained only 2% of the total variation (Table 3.2). No interaction between both factors was found for the number of aborted eggs as well (two-way ANOVA, $F_{4,45} = 1.7$, $p>0.05$). The slightly increase in the number of neonates in Dph_CBZ in response to CBZ concentrations might be a compensatory mechanism. This may be related to the r-strategy where more neonates are produced, usually with lower sizes, when under unfavourable conditions. This is usually explained by the lower amount of resources allocated to offsprings, which decreases (per neonate) with the increasing number of neonates, leading to an increased sensitivity towards unfavourable conditions (Smith and Fretwell, 1974). For F5 *D. magna,* their length after 21 days showed no significant differences between both Dph_Clean and Dph_CBZ populations (two-way ANOVA, $F_{1,45} = 2.7$, $p>0.05$) and no interaction between both factors (two-way ANOVA, $F_{4,45} = 1.8$, $p>0.05$) (Fig. 3.5c and Table 3.2).

In the F11 generation, significant effects of CBZ were detected for the number of neonates (two-way ANOVA, $F_{4,41}= 93.2$, $p<0.001$) and for the number of aborted eggs (two-way ANOVA, $F_{4,41} = 16.6$, $p<0.001$) (Fig. 3.5a and 3.5b and Table 3.2). Neonates from this generation showed a similar pattern of response in terms of reproductive output when compared to F5 neonates, where similar EC_{50} values (Fig. 3.4a) were obtained for Dph_Clean (31.7 µg/L, SE 2.4) and Dph_CBZ (34.6 µg/L, SE 1.1) populations ($p>0.05$). Although a higher number of Dph_CBZ neonates was observed (except for the 50 µg/L CBZ treatment) (Fig. 3.5a'), no significant differences were attained between both populations (two-way ANOVA, $F_{1,41} = 3.4$, $p>0.05$) (Fig. 3.5a' and Table 3.2). No interaction between both factors was found (two-way ANOVA, $F_{4,41} = 1.6$, $p>0.05$). The same occurred for the aborted eggs with no significant differences between both populations (two-way ANOVA, $F_{1,41} = 0.3$, $p>0.05$) and no interaction between both factors (two-way ANOVA, $F_{4,41} = 1.0$, $p>0.05$) (Fig. 3.5b' and Table 3.2). The pattern of aborted eggs was similar between F5 and F11 in Dph_Clean, however for Dph_CBZ there was an overall decrease in the number of aborted eggs from F5 to F11 (except in the 5 µg CBZ/L concentration) (Fig. 3.5b and 3.5b'). Daphnid's length showed no differences between the Dph_Clean and Dph_CBZ populations (two-way ANOVA, $F_{1,41} = 0.005$, $p>0.05$) and no interaction between both factors was found (two-way ANOVA, $F_{4,41} = 0.4$, $p>0.05$) (Fig. 3.5c'). Overall, length did not appear to be a sensitive endpoint in the present study.

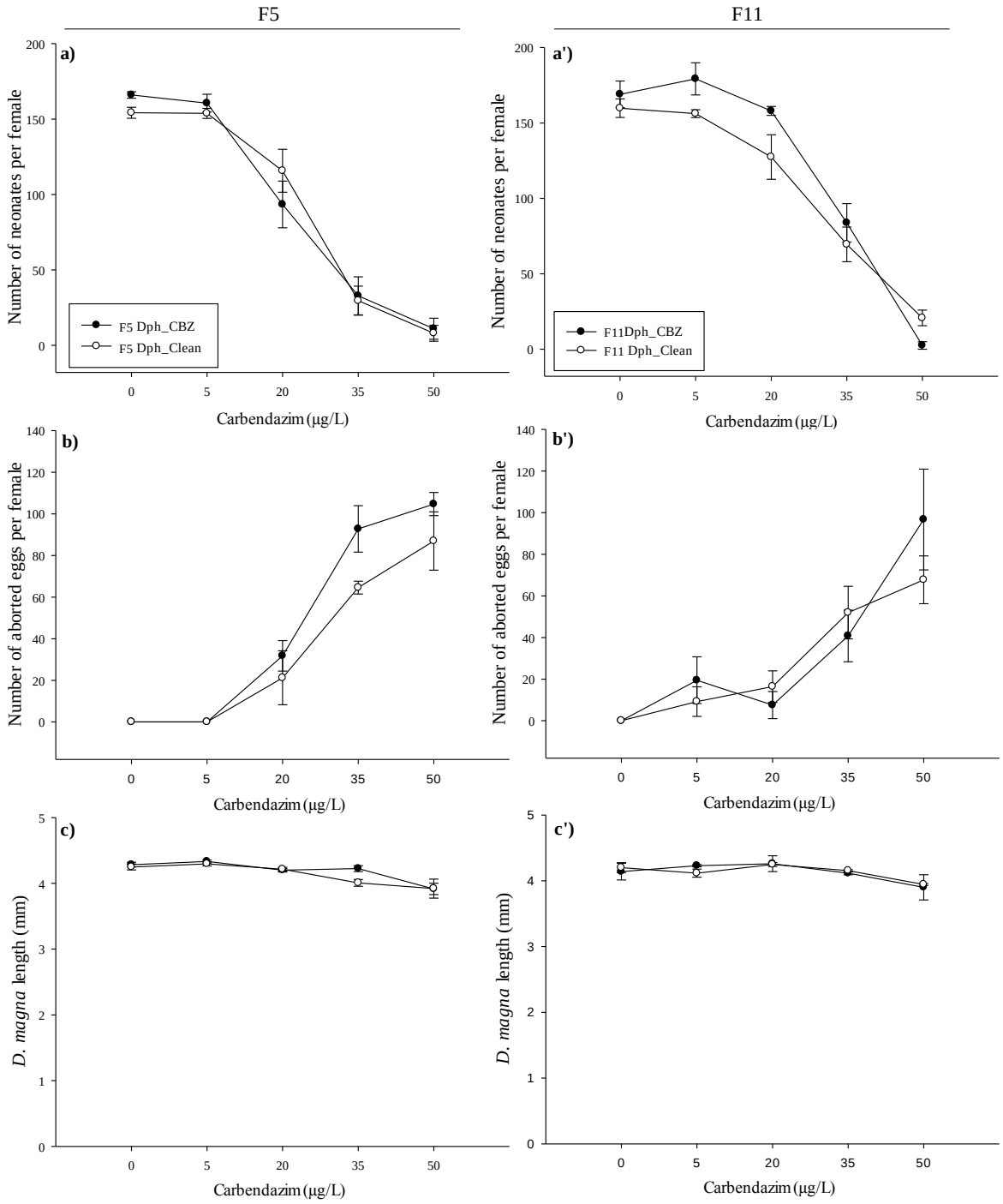

Figure 3.5. Effects on the reproduction effort (**a** and **a'**) and (**b** and **b'**) and length (**c** and **c'**) of F5 and F11 *Daphnia magna* exposed to carbendazim for 21 days, for Dph_Clean (white dots) and Dph_CBZ (black dots). Data are expressed as mean values and standard error.

Table 3.2. Two-way ANOVA results testing for effects of populations of *Daphnia magna* (Dph_Clean *vs.* Dph_CBZ), carbendazim exposure ([CBZ]) and their interaction on the reproduction output in the F5 and F11 generation.

	DF	Sum of squares	F	*p*-value	R^2
F5 Reproduction (number of neonates)					
[CBZ]	4	172561.1	108.2	**<0.001**	0.91
Population	1	1.9	0.005	0.95	$1.0x10^{-5}$
[CBZ] x Population	4	1594.5	1.0	0.42	0.008
F5 Reproduction (number of aborted eggs)					
[CBZ]	4	69509.6	85.3	**<0.001**	0.86
Population	1	1429.4	7.0	**0.01**	0.02
[CBZ] x Population	4	1423.0	1.7	0.161	0.02
F5 Length					
[CBZ]	4	0.8	16.4	**<0.001**	0.61
Population	1	0.03	2.7	0.11	0.02
[CBZ] x Population	4	0.08	1.8	0.16	0.06
F11 Reproduction (number of neonates)					
[CBZ]	4	149094.5	93.2	**<0.001**	0.91
Population	1	1360.5	3.4	0.74	0.008
[CBZ] x Population	4	2614.0	1.6	0.19	0.02
F11 Reproduction (number of aborted eggs)					
[CBZ]	4	36195.6	16.6	**<0.001**	0.64
Population	1	144.6	0.3	0.61	0.003
[CBZ] x Population	4	2124.1	1.0	0.43	0.04
F11 Length					
[CBZ]	4	0.46	4.2	**0.008**	0.33
Population	1	0.0001	0.005	0.95	$7.1x10^{-5}$
[CBZ] x Population	4	0.04	0.4	0.81	0.03

To evaluate the DNA damage in the reproduction test, F5 neonates from Dph_Clean and Dph_CBZ were exposed to control conditions, 5 µg/L and 20 µg/L of CBZ for 13 days. During this period, when their 3rd brood was released, these neonates were pooled and DNA damage was assessed. CBZ caused a significant effect in DNA damage, with increase in DNA damage (%) with increasing concentrations (two-way ANOVA, $F_{2,23}$ = 74.2, $p<0.001$) (Fig. 3.6 and Table 3.3). DNA damage significantly differed between the two populations Dph_Clean and Dph_CBZ, with Dph_CBZ presenting always a higher percentage of DNA damage (two-way ANOVA, $F_{1,23}$ = 12.0, $p<0.05$) (Fig. 3.6 and Table

3.3). However, no significant interaction was found between the two factors (two-way ANOVA, $F_{2,23} = 1.45$, $p>0.05$) (Table 3.3).

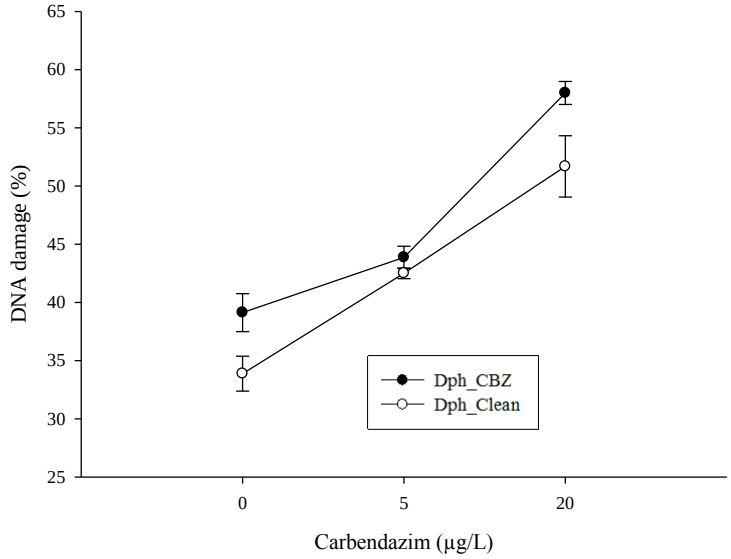

Figure 3.6. DNA damage (in %) in cells of *Daphnia magna* neonates (3rd brood) born from F5 exposures to control medium, 5 and 20 µg/L of carbendazim, for Dph_Clean (white dots) and Dph_CBZ (black dots). Data are expressed as mean values and standard error.

Table 3.3. Two-way ANOVA results testing for effects of populations of *Daphnia magna* (Dph_Clean *vs.* Dph_CBZ), carbendazim exposure ([CBZ]) and their interaction on the percentage of DNA damage in the F5 generation.

	DF	Sum of squares	F	*p*-value	R^2
F5 DNA damage (%)					
[CBZ]	2	1378.9	74.2	**<0.001**	0.82
Population	1	111.6	12.0	**0.003**	0.07
[CBZ] x Population	2	27.0	1.45	0.26	0.02

The intrinsic rate of natural increase (*r*) was calculated for F5 and F11 daphnids of both populations exposed for 21 days to a gradient of CBZ concentrations. The EC_{50} values for this endpoint were similar between Dph_Clean and Dph_CBZ in the F5 generation: 37.9 µg/L (SE 1.1) and 37.5 µg/L (SE 3.5), respectively ($p>0.05$), as well as in the F11 generation: 43.9 µg/L (SE 1.7) and 41.7 µg/L (SE 2.3) for Dph_Clean and Dph_CBZ, respectively ($p>0.05$) (Fig. 3.4b).

Although this similar pattern of response was obtained for both populations, a drastic effect of CBZ on the *r* values was observed while testing both F5 and F11 generations from both populations (Fig. 3.7a and 3.7b), with significant effects of CBZ detected for the F5 (two-way ANOVA, $F_{4,46}$ = 88.0, p<0.001) and for the F11 generation (two-way ANOVA, $F_{4,46}$ = 42.2, p<0.001) (Table 3.4). This is mainly caused by a reduction in the number of neonates produced per *Daphnia*, as well as a reduction of the number of broods. In the reproduction tests, F5 and F11 daphnids from both isoclonal populations showed similar *r* values in control treatments. The same occurred in the lowest concentration (5 µg/L) with similar *r* values except in the F11 Dph_CBZ, where an increase in the *r* value was observed. In the F11, Dph_CBZ consistently presented a higher *r* value compared with Dph_Clean except at the highest concentration (Fig. 3.7b). This pattern at the highest concentration could be related to the strong toxic effects caused to daphnids. No significant differences were observed between the responses of Dph_Clean and Dph_CBZ populations to CBZ in terms of *r* for F5 (two-way ANOVA, $F_{1,46}$ = 0.09, p>0.05) nor for F11 (two-way ANOVA, $F_{1,46}$ = 0.15, p>0.05) (Table 3.4). No interaction between both factors were observed for F5 (two-way ANOVA, $F_{4,46}$ = 1.57, p>0.05) nor for F11, indicating that populations responded similarly to CBZ concentrations (two-way ANOVA, $F_{4,46}$ = 1.11, p>0.05) (Table 3.4).

Dph_CBZ population did not show any signs of evolution towards tolerance even after eleven generations (F11) of exposure. This conclusion can be made because a similar decline in the reproductive output was found with increasing concentrations of CBZ for both Dph_CBZ and Dph_Clean populations. Only few studies are available where the intrinsic rate of natural increase (r) is assessed in successive generations. In the study of Zalizniak and Nugegoda (2006), the effects of chlorpyrifos were tested in three successive generations of Daphnia carinata, showing no changes in the intrinsic rate of natural increase.

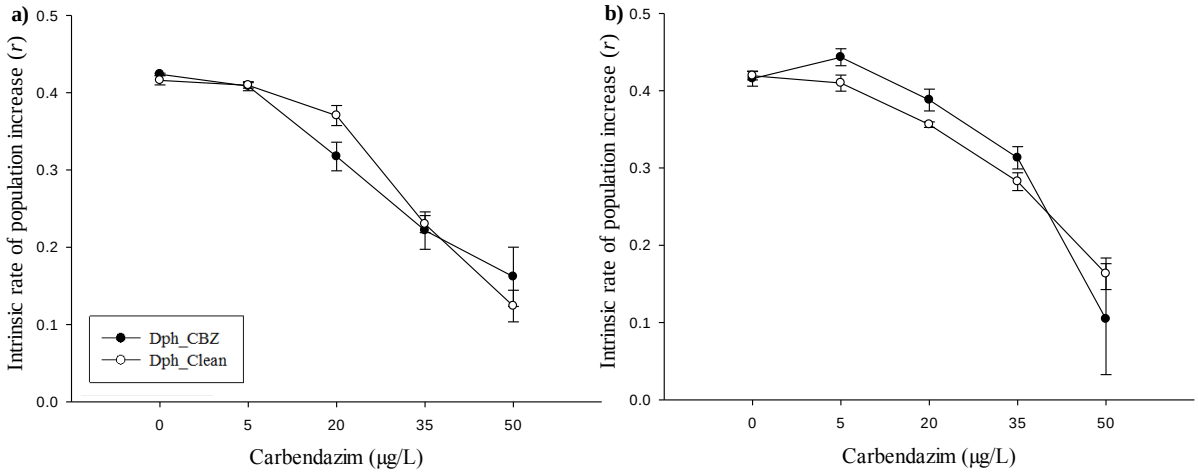

Figure 3.7. Intrinsic rate of population increase (*r*) for a 21 day exposure to carbendazim in *Daphnia magna* **a)** F5 and **b)** F11 generations, for Dph_Clean (white dots) and Dph_CBZ (black dots). Data are expressed as mean values and standard error.

Table 3.4. Two-way ANOVA results testing for effects of populations of *Daphnia magna* (Dph_Clean *vs.* Dph_CBZ), carbendazim exposure ([CBZ]) and their interaction on the intrinsic rate of natural increase (*r*) in the F5 and F11 generation.

	DF	**Sum of squares**	**F**	***p*-value**	**R^2**
F5					
Intrinsic rate of natural increase (*r*)					
[CBZ]	4	0.54	88.0	**<0.001**	0.89
Population	1	0.0001	0.09	0.77	$1.6x10^{-4}$
[CBZ] x Population	4	0.0097	1.57	1.57	0.02
F11					
Intrinsic rate of natural increase (*r*)					
[CBZ]	4	0.58	42.2	**<0.001**	0.81
Population	1	0.0005	0.15	0.70	$7.0x10^{-4}$
[CBZ] x Population	4	0.015	1.11	0.36	0.02

3.3.3 Feeding inhibition tests

A feeding inhibition test was performed with F6 juveniles from the Dph_Clean and Dph_CBZ populations. Exposure to CBZ significantly reduced the feeding rate after 24h exposure (two-way ANOVA, $F_{5,35}$ = 118.3, p<0.001) and the feeding rate in the 4h post-exposure of daphnids (two-way ANOVA, $F_{5,35}$ = 188.6, p<0.001) for both populations (Table 3.5). For the 24h exposure, the EC_{50} value obtained for CBZ exposures of Dph_Clean was 385.1 µg/L (SE 33.1) and for Dph_CBZ it was >450 µg/L (Fig. 3.4c). The

feeding rate (24h exposure) on the Dph_CBZ population differed from the feeding rate (24h) on Dph_Clean population (two-way ANOVA, $F_{1,35} = 40.9$, $p<0.001$) and there was a significant interaction between both factors (two-way ANOVA, $F_{5,35} = 4.3$, $p<0.05$) (Fig. 3.8a and Table 3.5). Feeding rates (24h exposure) on the Dph_CBZ were generally higher compared with Dph_Clean (except in the first concentration: 50 µg CBZ/L). This overall increase in the feeding rates in Dph_CBZ might represent an attempt to overcome stress (in this case caused by CBZ). This increase in feeding activities also requires energy that afterwards might not be allocated for other processes (*e.g.* reproduction or detoxification mechanisms) (Congdon *et al.*, 2001; Minguez *et al.*, 2015).

Once daphnids were transferred to clean medium (corresponding to the 4h post-exposure period), despite the similar post-exposure EC_{50} values (129.1 µg/L (SE 8.9) for Dph_Clean and 168.5 µg/L (SE 28.2) for Dph_CBZ, $p>0.05$) (Fig. 3.4d), feeding rates were again different between the two populations (two-way ANOVA, $F_{1,35} = 21.1$, $p<0.001$), with higher feeding rates in Dph_CBZ when compared to Dph_Clean (Fig. 3.8b and Table 3.5). Both factors significantly interacted (two-way ANOVA, $F_{5,35} = 3.5$, $p<0.05$) (Table 3.5), indicating that populations responded differently to the concentrations of CBZ. Few studies exist regarding the feeding behaviour in different generations of *D. magna*. Villarroel and colleagues (1999) evaluated the effect of tetradifon on the feeding rates of daphnids in four different generations. The authors observed that the toxic effect of tetradifon was greater in F1 and F3 generation than in the F0 generation. In addition, the ability of daphnids to recover was evaluated by assessing the feeding rates of F3 neonates after being transferred to clean water, still, their feeding rates were reduced when compared to their F0 feeding activities. The authors postulated that tetradifon could be transferred (bioaccumulated) from mothers to neonates of the next generations (F1 and F3). However, recovery is related to time and longer permanence in clean medium, which could promote chemical excretion, therefore reducing the effects in the organisms (Villarroel *et al.*, 1999).

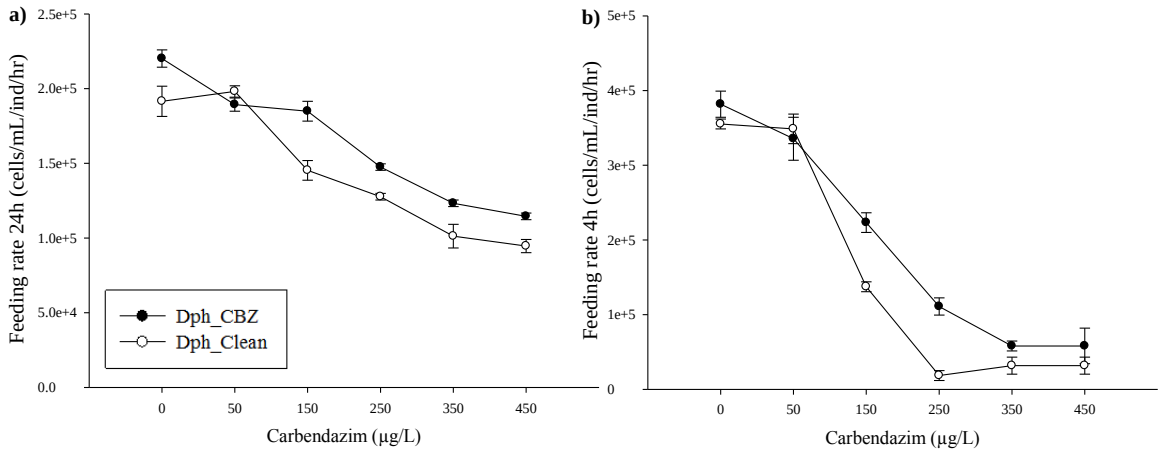

Figure 3.8. Feeding rates (cells/mL per daphnia per hour) of F6 *Daphnia magna* exposed to carbendazim for **a)** 24h and after a **b)** 4h post-exposure period in clean medium, for Dph_Clean (white dots) and Dph_CBZ (black dots). Data are expressed as mean values and standard error.

Table 3.5. Two-way ANOVA results testing for effects of populations of *Daphnia magna* (Dph_Clean *vs.* Dph_CBZ), carbendazim exposure ([CBZ]) and their interaction on the feeding rate 24h and 4h exposure in the F6 generation.

	DF	**Sum of squares**	**F**	***p*-value**	**R^2**
F6 Feeding rate (24h)					
[CBZ]	5	53032064323.2	118.3	**<0.001**	0.87
Population	1	3665196704.9	40.9	**<0.001**	0.06
[CBZ] x Population	5	1939828074.2	4.3	**0.006**	0.03
F6 Feeding rate (4h)					
[CBZ]	5	668470085578.4	188.6	**<0.001**	0.94
Population	1	14928558215.3	21.1	**<0.001**	0.02
[CBZ] x Population	5	12355044892.6	3.5	**0.016**	0.02

From the previous feeding inhibition tests, looking at the post-exposure phase, where daphnids were left to feed for 4h under clean medium, it was observed that those from Dph_CBZ still had a decreased feeding activity (Fig. 3.8b). Within this experimental setup, daphnids from Dph_CBZ were tested in CBZ exposure and in the control treatments, and they showed consistently higher feeding rates compared to the Dph_Clean population under the same exposure trials (Fig. 3.8a and 3.8b). This might indicate that, although they

stayed for 24h under clean medium exposure (control treatments), daphnids were still recovering from the multigenerational exposure to CBZ, leading to higher feeding rates.

4. Conclusions

The present study demonstrated different patterns regarding daphnids' sensitivity due to CBZ exposure throughout 12 generations. At the individual level, these differences were mainly observed in daphnids feeding activity, which is also considered a functional endpoint. The population of daphnids exposed to CBZ increased their feeding activity (comparing with the control population), and this could be interpreted as a compensatory mechanism of daphnids in consequence of stress. Looking at the immobilisation and reproduction, these two endpoints seemed to be somehow compensated throughout generations and no clear effects were observed. Although the toxicity effects towards CBZ exposure was sometimes similar between unexposed daphnids and daphnids that were continuously exposed to CBZ, DNA damage increased throughout generations in a cumulative way. This DNA damage might have consequences after exposure to other stressors and those effects are unpredictable. Daphnids reproduction, growth and survival levels were maintained possibly at expenses of energy obtained from their higher feeding rates. However, this was not enough to repair DNA damages and genotoxic effects were attained. This might be related with the fitness costs hypothesis, which was already reported for daphnids (Agra et al., 2010).

Acknowledgments

This work was supported by the project RePulse- Responses of *Daphnia magna* exposed to chemical pulses and mixtures throughout generations (FCOMP-01-0124-FEDER-019321; Refª. FCT PTDC/AAC-AMB/117178/2010), by funding FEDER through COMPETE- Programa Operacional Factores de Competitividade, and by the Portuguese Science Foundation (FCT) through CESAM: UID/AMB/50017/2013. Ana Rita Rego Gouveia Silva was funded by the project "MARPRO- Conservation of marine protected species in Mainland Portugal" through the doctoral fellowship (BD/UI88/5534/2011), Diogo Cardoso was funded by the doctoral grant (PD/BD/52569/2014), Andreia Cruz was funded by an individual post-doctoral grant (BPD/UI88/2886/2013), within the project "Sustainable Use of Marine Resources" - MARES (CENTRO-07-ST24-FEDER-002033), financed by QREN, Mais Centro- Programa Operacional Regional do Centro e União Europeia/Fundo Europeu de Desenvolvimento Regional and João Pestana was funded by an individual post-doctoral grant (SFRH/BPD/45342/ 2008).

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Supplementary data

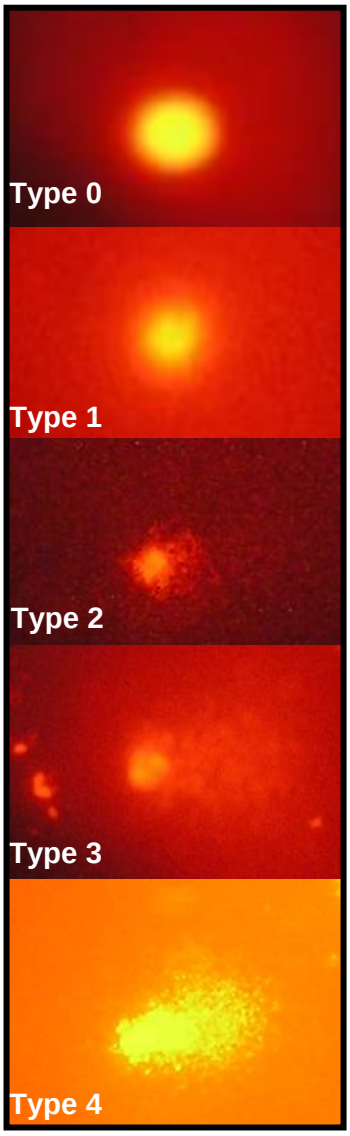

Figure 3.1 SD. Comet type scale used in daphnid cells.

Chapter 4

Long-term exposure of Daphnia magna and potential responses along mixture pulses

Long-term exposure of *Daphnia magna* and potential responses to chemical pulses

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Abstract

Aquatic organisms might be exposed episodically or continuously to chemicals for long-term periods throughout their lifespan. Pesticides widely used in agriculture can appear in surface waters mainly through runoffs, representing a potential hazard to aquatic organisms. In addition, several chemicals may be present simultaneously in the environment or as pulses, being difficult to predict accurately how their joint effects will take place.

Therefore, the aim of the present study was to investigate how *Daphnia magna* (clone k6) exposed throughout generations to a model pesticide (the fungicide carbendazim) would react upon pulse exposures to other chemicals (triclosan) and the mixture of both chemicals (carbendazim and triclosan). Within this, *D. magna* were continuously exposed to an environmental relevant concentration of carbendazim (5 µg/L) for 12 generations and at F8 and F12 generations daphnids were exposed to pulses of triclosan and to pulses of the binary mixture of triclosan and carbendazim. To assess the induced effects of pulses, responses of daphnids continuously exposed to carbendazim and kept in clean medium were compared under an immobilisation test and using the comet assay (DNA integrity).

After 12 generations (F12), carbendazim exposed daphnids appeared to have similar sensitivity to triclosan (similar 48h-LC_{50} values), when compared to daphnids in clean medium. However, F12 daphnids previously exposed to carbendazim presented an overall higher percentage of DNA damage when compared to those in clean medium, after being also exposed to a range of concentrations of carbendazim and triclosan, and to their binary combination. The binary mixture toxicity patterns observed were generally similar for daphnids in clean medium and daphnids exposed to carbendazim for the immobilisation and DNA damage data.

Key words: *Daphnia magna*, multigenerations, carbendazim, triclosan, chemical pulses, DNA damage

1. Introduction

In the environment, aquatic organisms might be exposed not only to one or more chemicals (mixtures), but also to pulses of other chemical(s) from different sources (Kuster *et al.*, 2008; Solomon *et al.*, 1996). Chemical exposure may be short-termed (*e.g.* as pulses), last for the entire lifetime of an organism, or even for several generations. Environmental chemical inputs might be related to pesticide seasonal patterns of application in agriculture, from waste water treatments plants (*e.g.* effluents) or from other accidental or deliberate chemical discharges (McCahon and Pascoe, 1990; Solomon *et al.*, 1996). A continuous chemical exposure, even at low concentrations, might have consequences to organisms' fitness, originating more or less sensitive organisms. These changes in organisms' sensitivity can be evaluated by testing their sensitivity toward (other) chemical exposure. Considering this, multigenerational studies can be of extremely importance as they might provide estimation for population effects and helping on the risk assessment process of chemicals in the aquatic system. *D. magna* is a suitable test organism for effects' evaluation throughout several generations as it reproduces by parthenogenesis (with no recombination), eliminating confounding genetic differences, and possibly making this species more susceptible to DNA damage throughout time (Harris *et al.*, 2012; Hebert and Ward, 1972; Simon *et al.*, 2003; Sukumaran and Grant, 2013). *Daphnia* standardized tests are often advised for regulatory purposes and the comet assay can be used as a complement to evaluate the DNA damage in single cells as an early warning tool. This DNA damage, expressed as DNA strand breaks, have been reported as sensitive biomarker of genotoxicity (Collins *et al.*, 1997; Singh *et al.*, 1988).

Currently there is not a complete picture on how organisms behave under long-term exposures, and the majority of tests are carried out throughout two or three generations (Brausch and Salice, 2011; Brennan *et al.*, 2006; Massarin *et al.*, 2010). Therefore increasing exposure time and adding extra stressors to the testing systems may straighten the knowledge gap that exists. Considering this, the aim of the present study was to investigate how *D. magna* exposed throughout generations to a model chemical would react upon pulse exposures to other chemicals and the mixture of both chemicals (the pre-

exposed and the new chemical). For that the fungicide carbendazim (CBZ) (methyl-2-benzimidazole carbamate), largely used in agriculture (WHO, 1993) was chosen as model chemical for the continuous exposure. CBZ has been used in different crops, therefore it is likely to be continuously released during some months (EU Pesticide Database, 2015). CBZ can appear in aquatic systems due to agricultural fields' runoffs and it has already been detected at concentrations of approximately 5 µg/L in surface waters (Chatupote and Panapitukkul, 2005; Palma *et al.*, 2004). Therefore, in the experimental design, *D. magna* organisms were continuously exposed to 5 µg/L of CBZ throughout twelve generations and triclosan (TCS) (5-Chloro-2-(2,4-dichlorophenoxy)phenol) was chosen as the pulse chemical due its worldwide use as antimicrobial agent, in many personal care products (Brausch and Rand, 2011). When TCS enters the waste water treatment plants (WWTP), its complete removal can be inefficient, which can lead to its release to the environment (Bester, 2003). A few years ago, a USA river receiving a treated wastewater discharge from a manufacturing plant presented TCS concentrations up to 6-14 mg/L (NICNAS, 2009). In Portugal, TCS was already detected in urban wastewater samples, although at lower concentrations: 0.124 µg/L (Neng and Nogueira, 2012). Despite the different sources/origins of CBZ and TCS, there is a possibility of their co-occurrence in surface waters. Therefore, studies predicting joint effects of chemicals from different sources, which are usually disregarded, can provide information to derive more accurately risk assessment. To attain effects on *D. magna*, organisms previously exposed to CBZ for several generations (F8 and F12) and from clean medium (control), were exposed to pulses of TCS and mixture pulses of both chemicals, along with the sensitivity testing to CBZ. Survival and DNA damage were reported based on results from immobilisation tests and from the comet assay.

2. Materials and methods

2.1 Test organism

D. magna Straus clone K6 (originally from Antwerp, Belgium) were obtained from laboratory cultures maintained at the University of Aveiro (Portugal), in ASTM moderated-hard-water medium (American Society for Testing and Materials) (ASTM, 1980), at temperature between 19ºC and 21ºC and a 16h light-8h dark photoperiod. *D.*

magna cultures consisted of 1 L glass vessel containing culture medium and 25 daphnids. The medium was renewed three times a week and daphnids were fed with *Raphidocelis subcapitata* (formerly known as *Pseudokirchneriella subcapitata*) at a concentration of $3x10^5$ cells/mL and supplemented with an organic extract (Marinure seaweed extract, supplied by Glenside Organics Ltd.).

2.2 Test chemicals

CBZ (CAS No. 10605-21-7, 99.4% purity, Bayer) and TCS (Irgasan, CAS No. 3380-34-5, 97% purity, Sigma-Aldrich) were used to perform ecotoxicity tests in the present study. For the multigenerational test, a stock solution of CBZ was prepared in ASTM medium; for the immobilisation tests and daphnids' exposures for the comet assay stock solutions were prepared in ASTM with acetone due to the low solubility of TCS and CBZ at higher concentrations. A solvent control of 100 µL acetone/L was also included in all experimental setups as recommended by the OECD guideline 23 (OECD, 2000). Chemical analyses were performed to achieve real concentrations of TCS and CBZ in the test medium at Marchwood Scientific Services, Southampton, UK. For that, extra beakers were used, one with ASTM medium contaminated with CBZ and another with ASTM medium contaminated with TCS. The analyses for TCS were performed by Gas Chromatography-Mass Spectrometry (GCMS-MS). A representative portion of the samples (200-300 mL) was extracted with 20 mL of acetonitrile (containing 1% acetic acid). Samples were then subjected to a solid phase extraction stage using a 200 mg cartridge. A methanol wash followed and 10 µl final injection volume applied. Standards were prepared in solvents at seven levels with recoveries in the range 70-120%. The analyses for CBZ were performed by Liquid Chromatography-Mass Spectrometry (LCMS-MS) using the Querchers method. A representative portion of the sample (200-300 mL) was extracted with 20 mL of acetonitrile (containing 1% acetic acid). This was followed by a partitioning step with magnesium sulphate and a subsequent buffering step with sodium acetate. After mixing an aliquot with methanol, the extract was injected directly into the LCMS-MS system (instrument Agilent 6410 Triple Quad LCMs-MS) without any clean-up. A 10 µl injection volume was utilized. Standards were prepared in solvents at seven levels with recoveries in the range 70-120%. To determine chemical decay in time, the degradation constant (*k0*) was calculated by the following equation:

(Equation 1) $C_t = C_0 e^{-k0t}$

Where C_0 corresponds to the initial external concentration (µg/L), k_0 corresponds to the constant of degradation of the chemical in the medium (/hour) and t corresponds to time (hours) (Widianarko and Van Straalen, 1996).

2.3 Multigenerational experimental setup

An isoclonal population of *D. magna* was exposed continuously to 5 µg/L of CBZ (Dph_CBZ). This concentration (5 µg CBZ/L) was based on previous results where a NOEC (no observed effect concentration) for reproduction of 5 µg/L was derived (Chapter 2 - Silva *et al.*, 2015) and also representing an environmental relevant concentration (Palma *et al.*, 2004). An isoclonal population of daphnids in clean medium (Dph_Clean) was kept simultaneously. Both populations were kept in ASTM hard water, fed with *R. subcapitata*, and supplemented with organic extract (Marinure seaweed extract, supplied by Glenside Organics Ltd.), differing only on the presence/absence of CBZ. Medium renewal was performed as described for the culture procedures. The experimental setup included only one vessel per population with 25 daphnids each as it was aimed to increase efforts on the generations' number and neonates' fitness testing (see beneath) in detriment of replicates. Since daphnids are clonal organisms the genetic variation within replicates may be lower than for other organisms.

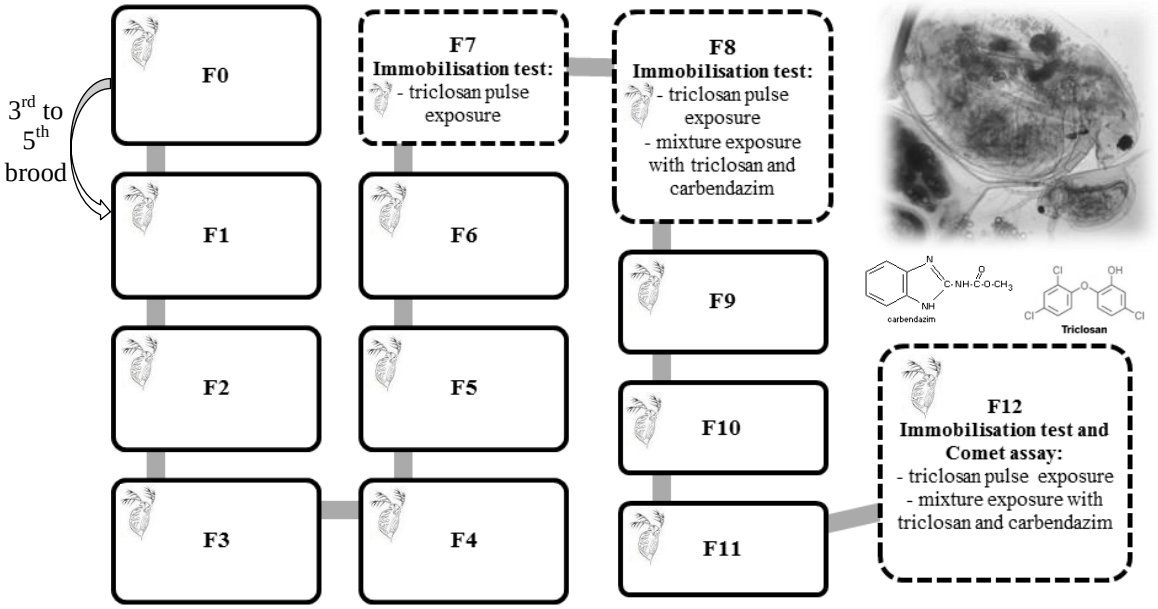


Figure 4.1. Experimental design of the multigenerational setup with two isoclonal populations of *Daphnia*

magna: continuous exposed to carbendazim (Dph_CBZ) and daphnids in clean medium (Dph_Clean). Each box represents one of the 12 generations for both populations and the respective tests carried out (dashed line boxes).

Neonates from the third to fifth brood (<24 h old) were used to start the next generation exposure for both populations, using always the same brood for Dph_Clean and Dph_CBZ isoclonal populations. To control differences in daphnids' responses due to sensitivity variations in organisms, in each testing generation, both immobilisation tests and comet assays were performed simultaneously using Dph_Clean and Dph_CBZ populations (Loureiro *et al.*, 2010). The experimental design is schematized in Figure 4.1.

At the end of the multigenerational experiment, neonates from the F12 generation from Dph_CBZ were transferred to clean medium for 24h, in order to understand whether they were able to recover at a subcellular level. Three replicates, with fifteen juveniles each were used and processed to run the Comet assay, based on the methodology developed by Nogueira *et al.* (2006). Positive controls consisted of daphnid's cells previously exposed to hydrogen peroxide (H_2O_2). To prevent UV-induced DNA damage, the comet assay procedures were conducted under yellow light. Briefly, organisms were placed in 1.5 mL *Eppendorfs* containing 1 mL of phosphate-buffered saline (PBS), 10% dimethyl sulfoxide (DMSO) and 20 µM ethylenediamine tetra-acetic acid (EDTA). Organisms were gently disintegrated mechanically with an appropriate. Samples were centrifuged (200 *g*) during 10 min at 4ºC and most of the supernatant was removed gently. From the pellet (containing cells), 10 µL were transferred to *Eppendorfs* containing 0.5% low melting point agarose (at 37ºC). To uniformly distribute this mixture on the microscope glass slides, containing 1% normal melting agarose, the coverslips were applied. Slides were placed on ice for 10 min. Following that and after the removal of the coverslips, slides were placed, for at least 1h, in a solution of 10 mM Tris-HCl, 100 mM EDTA, 2.5 M NaCl, 10% DMSO, 10% Triton X-100, pH 10, for cell lysis. For the electrophoresis a solution with 10 M NaOH, 200 mM Na_2-EDTA, and distilled water was used. Slides were placed in the electrophoresis tin for 15 min before starting the electrophoresis, allowing the DNA to denature and unwind. Then, an electric current of 300 mA (30 Volts) was applied for 10 min. For neutralization, slides were washed with 0.4 M Tris-HCl (pH=7.5) and then dehydrated with absolute ethanol 100% for 10 sec and finally left to dry for 1 day in the dark.

For the image analysis, slides were stained with 100 µL ethidium bromide (20 µL/mL), and then analyzed using a fluorescence microscope (Olympus BX41TF, China) at 400 x magnification. One hundred cells per slide were examined and the scorer was unaware of the treatment condition when reading the slides. DNA damage was visually scored: each cell was scored on a 0 to 4 scale, as described by Duthie and Collins (1997). Type 0 represents no DNA damage, type 1 and 2 represent mild to moderate damage, respectively, and type 3 and 4 represent extensive DNA damage. The total comet score was calculated according to the method of Duthie and Collins (1997): (number of cells in type 0 × (type) 0) + (number of cells in type 1 × (type) 1) + (number of cells in type 2 × (type) 2) + (number of cells in type 3 × (type) 3) + (number of cells in type 4 × (type) 4). Therefore, the total score for 100 cells could range between 0 (all comets with no damage) to 400 (all comets with maximum damage). A percentage of DNA damage was calculated. Fig. 4.1 SD represents a comet type scale in daphnid cells.

2.4 Pulse and mixture testing: immobilisation and DNA damage

Immobilisation tests with TCS simulated short-term pulses of the chemical and were carried out in the generations F7, F8 and F12; while the effects of pulses of the binary mixture with CBZ and TCS were tested at F8 and F12 generations, using neonates originating from both populations Dph_Clean and Dph_CBZ (Fig. 4.1). In addition, a sensitive test with CBZ was also carried out within the mixture experimental setup.

Immobilisation tests were carried out accordingly to the OECD 202 guideline (OECD, 2004), with few adaptations. Daphnids with less than 24h were used to initiate the test, and after 24h and 48h, daphnids' immobilisation was recorded, after gentle agitation of the test beaker. Neonates were exposed to test solutions of CBZ, TCS and binary mixtures for 48h in 50 mL beakers with no food (16:8 h light-dark photoperiod and 20 ± 1^{0}C). The experimental setup consisted in a full factorial design (Fig. 4.2a) with three replicates of five neonates each, for every treatment and controls. Replicates were reduced in order to allow simultaneous testing (reducing effort) and to allow a reliable coverage of the exposure/response surface in the mixture trials by increasing the number of treatments and decreasing the replication. This procedure increases both reliability and power of the analysis, as the response surface analysis is based on a regression model (Loureiro *et al.*, 2010).

For the pulse exposure with TCS in F7, concentrations ranged from 400 to 1200 µg/L. For the single and mixture experiments with TCS and CBZ, in the F8 and F12 generation, neonates were exposed to concentrations ranging from 200 to 1000 µg/L of TCS and from 20 to 220 µg/L of CBZ corresponding to a minimum of 0.5 Toxic Units (TUs) and a maximum of 3 TUs of the mixture (where 1TU=LC_{50}) (Fig. 4.2a). Single chemical exposures referred above were performed simultaneously in each mixture test to account for the sensitivity variations throughout generations, but also to predict results that will be latter compared to the observed output (experimental data from the mixture exposure) (Loureiro *et al.*, 2010).

For the Comet assay, F12 neonates (with less than 24h) from both population Dph_Clean and Dph_CBZ (Fig. 4.1) were exposed to CBZ, TCS and their mixture for 24h. Three replicates, with fifteen juveniles each, were used for each control and concentrations. The comet assay was described above in detail. Neonates were exposed to 5, 20 and 25 µg/L of CBZ, representing a NOEC, LOEC (lowest-observed-effect concentration) and EC_{50} for daphnids' reproduction (Chapter 2 - Silva *et al.*, 2015). For TCS exposure, daphnids were exposed to 120, 160 and 206 µg/L of TCS, where the extremes represent the NOEC and EC_{50} for daphnids' reproduction, and 160 µg/L was chosen as a mean value concentration between the extremes (Chapter 2 - Silva *et al.*, 2015). Binary mixtures consisted on combinations of 120 µg/L of TCS and 5 µg/L of CBZ; 160 µg/L of TCS and 20 µg/L of CBZ; and 206 µg/L of TCS and 25 µg/L of CBZ (Fig. 4.2b), corresponding to a minimum of 1 TU and a maximum of 3 TUs (ΣTUs), respectively (Fig. 4.2b).

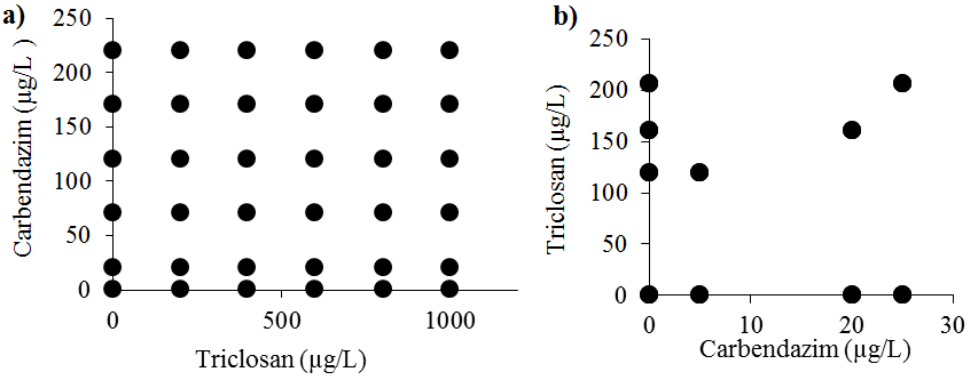


Figure 4.2. a) Full factorial design used for the immobilisation test in the F8 and F12 generation of *Daphnia*

magna **b)** Experimental design used for the comet assay in the F12 generation of *Daphnia magna* for the binary mixture exposures of carbendazim and triclosan.

2.5 Statistical Analysis

Data normality was assessed using the Shapiro-Wilk test and homoscedascity was assessed using Levene's equal variance test (Systat Software Inc., 2008).

In the immobilisation tests and the comet assays, differences between the negative control and the solvent control were checked using a t-test (Systat Software Inc., 2014). For the immobilisation data, the 48h-LC_{50} values were calculated using a nonlinear regression (equation: standard curves, four parameter logistic curve) (Systat Software Inc., 2014). LC_{50} values were statistically compared according to Sprague and Fogels (Sprague and Fogels, 1976).

For DNA damage, when possible, the 50% effective concentration (EC_{50}) was calculated using a nonlinear regression with a logistic function (Systat Software Inc., 2014). To detect differences between Dph_Clean and Dph_CBZ populations, for the DNA damage endpoint a two-way ANOVA with multiple comparisons examined by Holm-Sidak post hoc method was performed using SigmaPlot (Systat Software Inc., 2008), with chemical exposure and populations as fixed factors. The R-squared was calculated by dividing the sum of squares of each factor and of their interaction by the total sums of squares of the two-way ANOVAs (Hullett and Levine, 2003), to evaluate the percentage of variance accounted for each factor in the ANOVAs.

Data obtained in the mixture experimental setup were analyzed by comparing the obtained effects with the expected mixture effects based on the two conceptual models, Concentration Addition (CA) and Independent Action (IA). For that, the MIXTOX tool described by Jonker *et al.* (2005) was used. The CA model assumes that chemicals have the same modes of action (MoA) and they can be seen as dilution of one another and the IA model assumes that chemicals have different MoA, and therefore single chemical induced effects are independent. The IA model was the main conceptual model used in the present study because it is expected that from both chemical structure and mode of action they will behave differently inside the organism although potentially inducing specific similar effects. However, recently EFSA has released a report from the Scientific

Colloquium 21 on the "Harmonisation of Human and ecological risk assessment of combined exposure to multiple chemicals", which states that the mixture patterns should be predicted using the CA model as a precautionary approach in ecological approaches (EFSA, 2015), accordingly the CA model was used as well and was presented in supplementary data.

Deviations from these two models were attained for synergism (more severe effect) or antagonism (less severe effect), or more complex deviations: dose ratio (DR: deviations depending on the composition of the mixture) and dose level (DL: deviations differ at low or high doses of the chemicals) dependencies by adding two extra parameters, *a* and *b*. The biological interpretation of parameters, *a* and *b* is described on Table 4.1 and further explanation can be found in Jonker *et al.* (2005). The method of maximum likelihood was used to fit the data and the fundamental procedure to minimize the Sum of Squared Residuals (SS) was used by running the Solver Function in Microsoft® Excel. The best fit was chosen using 0.05 as the significance level.

The TUs approach was used, providing the contribution of each chemical to the toxicity mixture. TUs were calculated using the quotient $c_i/\mathrm{EC}x_i$, where c_i represents the individual concentrations of substances in the mixture and $\mathrm{EC}x_i$ denotes the effect concentration (Bliss, 1939; Jonker *et al.*, 2005; Loewe and Muischnek, 1926; Loureiro *et al.*, 2010).

Table 4.1. Interpretation of additional parameters, *a* and *b*, that define the functional from the deviation patterns from the reference models Concentration Addition (CA) and Independent Action (IA). Adapted from Jonker *et al.* (2005).

Deviation pattern	Parameter *a* (CA and IA)	Parameter *b* (CA)	Parameter *b* (IA)
Synergism/ Antagonism	*a*>0 antagonism *a*<0 synergism		
Dose-ratio dependent	*a*>0 antagonism except for mixture ratios where negative *b* value indicate synergism *a*<0 synergism except for mixture ratios where positive *b* value indicate antagonism	*bi*>0 antagonism where the toxicity of the mixture is caused mainly by toxicant *i* *bi*<0 synergism where the toxicity of the mixture is caused mainly by toxicant *i*	
Dose-level dependent	*a*>0 antagonism at low dose level and synergism at high dose level *a*<0 synergism at low dose level and antagonism at high dose level	b_{DL}>1 change at lower EC_{50} level b_{DL}=1 change at EC_{50} level *0*< b_{DL}<1 change at higher dose level than the EC_{50} b_{DL}<0 no change, but the magnitude is dose level dependent	b_{DL}>2 change at lower EC_{50} level b_{DL}=2 change at EC_{50} level *1*< b_{DL}<2 change at higher dose level than the EC_{50} b_{DL}<1 no change, but the magnitude is dose level dependent

3. Results and Discussion

3.1 Chemical analysis

The results on the chemical analysis have already been described elsewhere (Chapter 2 - Silva *et al.*, 2015). CBZ concentration in the ASTM medium decreased over time, with a decay rate (K_0) of 0.03/h (SE=0.005), showing that only 18% of the initial concentration of 7.2 µg/L remained after 48h. Regarding TCS the obtained decay rate (*K0*) was 0.06/h (SE=0.010), meaning that after 48h of the initial concentration of 165 µg/L only 1.3% of TCS concentration remained.

3.2 Multigenerational effects

Upon a multigenerational exposure to CBZ, a recovery did not seem to occur, because the exposed population had still a significant increase in DNA damage after being removed/maintained in clean medium for 24h, compared with the ones from clean medium (Dph_Clean) ($p<0.05$, Two-way ANOVA, Holm-Sidak method). The ability of daphnids to repair DNA damage (after exposure to benzo(a)pyrene and after a 9 days recovery period) has been reported, and is considered an important protection against genotoxic

compounds (Atienzar and Jha, 2004). Therefore, the extension of the recovery time (increase the period of time of daphnids in clean medium) could provide additional information, in order to evaluate if the chemical was excreted and eliminated (Villarroel *et al.*, 1999).

DNA damage induced by CBZ multigenerational exposures is described below. All control exposures in the ecotoxicity tests and comet assays from Dph_CBZ will represent effects from CBZ multigenerational exposures.

3.3 Pulse and mixture exposure: Immobilisation tests

In all the performed tests, no significant differences were observed between the solvent control and the negative control (t-test, $p>0.05$). In the statistical analysis, all comparisons between chemical treatments and the control group were carried out with the solvent control.

Some authors reported that *Daphnia* adapts to new environments in few generations, however what ensues after these generations is not usually studied and changes in daphnids' sensitivity might actually occur. After seven generations (F7), individuals from Dph_Clean were exposed to TCS and a 48h-LC_{50} of 539.3 µg/L (SE=6.5) was derived. When F7 offspring from Dph_CBZ was also exposed to TCS the 48h-LC_{50} showed a statistical similar value of 510.6 µg/L (SE=44.0) ($p>0.05$) (Fig. 4.3). In the F8 generation the 48h-LC_{50} for TCS were similar, with values of 595.5 µg/L (SE=9.5) and 605.3 µg/L (SE=0.2) respectively for Dph_Clean and Dph_CBZ (Fig. 4.3). For the F12 generation, the 48h-LC_{50} values were 693.2 µg/L (SE=n.d.) and 1040.3 µg/L (SE=423.4), respectively for Dph_Clean and Dph_CBZ (Fig. 4.3). The 48h-LC_{50} value for TCS was slightly higher for the Dph_CBZ comparing with the Dph_Clean population. No confidence intervals were derived and therefore the statistical comparison using the Sprague and Foguels formula was not carried out; yet both R^2 value derived were close to 1 (1 and 0.99, respectively). The pre-exposure to CBZ seem to not affect the organisms' response to TCS, showing an overall similar sensitivity between Dph_Clean and Dph_CBZ populations throughout the generations.

Although, the pulse exposure to another chemical may provide some information regarding cross-tolerance in the present work this does not seem to occur. In fact, no clear pattern towards tolerance was found also regarding the continuous exposure to CBZ,

neither cross-tolerance with TCS in the present work. Cross-tolerance, also referred as cross-resistance by some authors, occurs when one organism becomes tolerant to one chemical, and afterwards can become tolerant to another chemical due to, for instance, detoxification mechanisms that might confer tolerance against several chemicals (Georghiou, 1972). In the literature it is demonstrated that cross-resistance usually occurs when compounds have similar chemical structures and/or MoA (Oppenoorth, 1985). The chemical structures of CBZ and TCS are different, however the exactly mode of action in daphnids is unclear. Another factor contributing to a faster development of resistance is the selection pressure: the greater the pressure, the greater the degree of resistance (Crow, 1954). In the present study, the pressure might be considered low, since the concentration of CBZ in the continuous exposure throughout the generations of *D. magna* was low (5 µg/L). On the opposite, Brausch and Smith (2009) observed that cross-tolerance (referred by the authors as cross-resistance) occurred for both cyfluthrin and naphthalene using *D. magna*. The authors observed that daphnids resistant to cyfluthrin were also resistant to two additional insecticides: DDT (1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane) (compound with similar MoA) and methyl parathion (compound with different MoA). Cross-tolerance was evaluated by checking changes in 48h-LC_{50} values between F0 and F13 generation, representing a 2.1 and 2.6 times higher LC_{50} values respectively for DDT and methyl parathion (Brausch and Smith, 2009).

Besides the effects of a single chemical or mixture of chemicals to an organism, the duration of the exposure is of paramount relevance (Paumen *et al.*, 2008). This highlights the importance to study the toxicity effects in different generations and with different exposure times as responses to chemicals varies over time/generations.

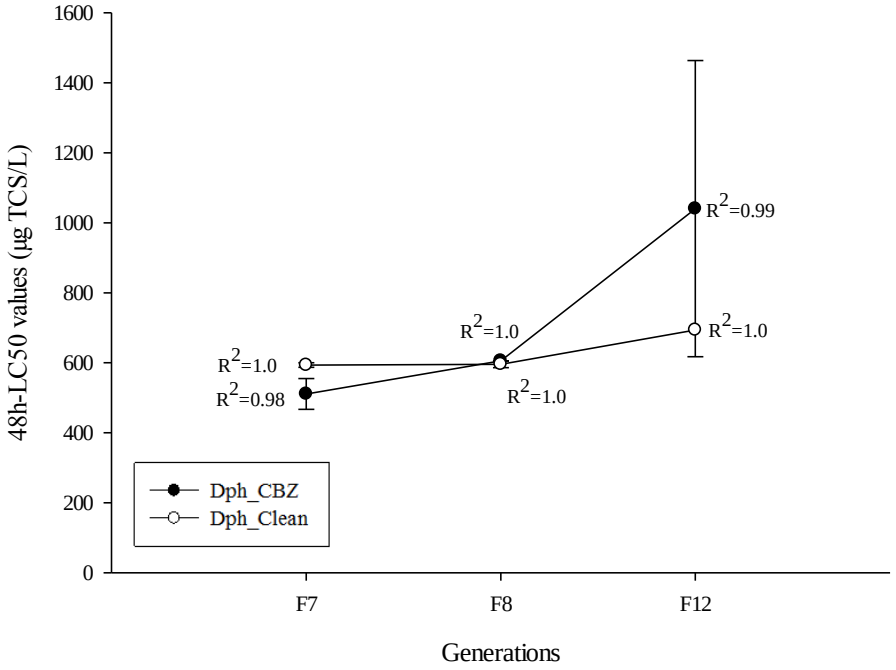


Figure 4.3. 48h-LC_{50} values derived from immobilisation tests with neonates from Dph_Clean (white dots) and Dph_CBZ (black dots) populations exposed to triclosan (TCS), at generations F7, F8 and F12. Data are expressed as 48h-LC_{50} value (µg/L) with standard error. *Dph_Clean *vs.* Dph_CBZ LC_{50} values for each generation, $p<0.05$.

CBZ acts on cell division inhibiting the reproduction capacity (Canton, 1976), increasing the number of aborted eggs, which is probably related with mitosis inhibition during eggs division in the brood pouch and also causes DNA damage (Ribeiro *et al.*, 2011; Chapter 2 - Silva *et al.*, 2015). TCS has been reported as inducing the activity of the antioxidant enzyme glutathione *S*-transferase (GST) in *D. magna*, and decreasing the superoxide dismutase (SOD) activity, which may indicate damage in the cell membranes (Peng *et al.*, 2013). It was also observed that TCS has the ability to induce DNA damage at concentrations starting in 120 µg/L in *D. magna* (Chapter 2 - Silva *et al.*, 2015). Bearing in mind that the specific mechanism of action of CBZ and TCS in daphnids is still vague, but assuming a potential difference between them in terms of chemical structure, to predict mixture toxicity effects the IA model was the main model used as a starting point. All CA modelling was also carried out and can be assessed in the supplementary data (Table 4.1 SD).

Considering the mixtures patterns of TCS and CBZ for the immobilisation data, it was found that data for Dph_Clean population in the F8 generation was significantly adjusted to the IA model (Table 4.2). When continuing the framework to assess potential deviations, antagonism was the best data fit detected (Table 4.2). The selection of this deviation is supported by the r^2 value, by the lowest SS value comparing with all the others deviations in the IA model and supported by the isobologram as well (Fig. 4.4 a1). Antagonism means that the effect of a mixture is less pronounced than the predicted based on individual chemical effects. For the F8 generation of neonates from Dph_CBZ population, the IA model fitted our data significantly with no further improvement by adding parameters for deviations (Table 4.2 and Fig. 4.4 b1). This means that, for daphnids previously exposed to CBZ (Dph_CBZ), the relative effect of one chemical remains unchanged in the presence of other chemical (Bliss, 1939), and an additivity of responses is achieved.

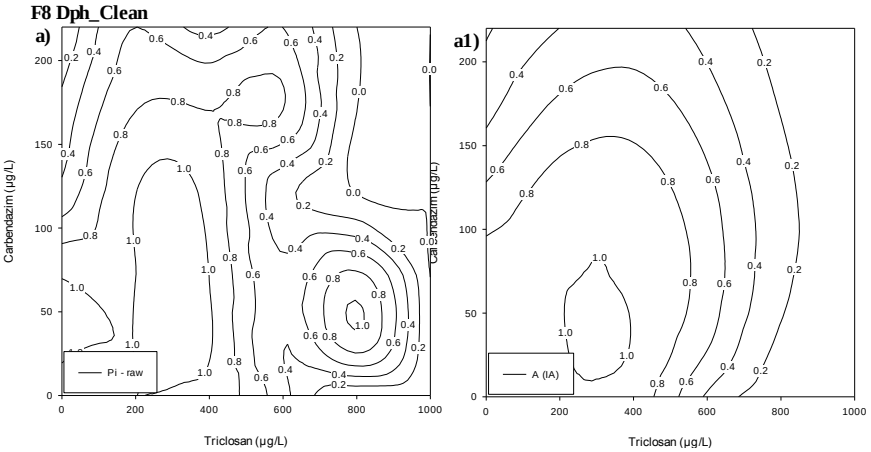
In the F12 generation, immobilisation data for Dph_Clean population was significantly adjusted to the IA model (Table 4.2). Continuing the nested framework for assessing potential deviations, the DL deviation showed the best fit (Table 4.2). For the DL deviation, the derived parameter *a* was positive meaning that there was an antagonism at low doses of both chemicals and synergism at high doses; parameter *b* was lower than one, providing the information that the change from antagonism to synergism would occur at higher concentrations than the tested ones (Table 4.1). Therefore, synergism was not observed in the isobologram and the main pattern for this endpoint was antagonism (Fig. 4.4 c1). In the F12 generation, the same deviation was observed for Dph_CBZ population, with a DL deviation with antagonism at low doses and synergism at high doses ($a>0$). The parameter *b* was again negative (Fig. 4.4d1). So, the pattern obtained and observed for F12 was also for antagonism (Table 4.2 and Fig. 4.4d1).

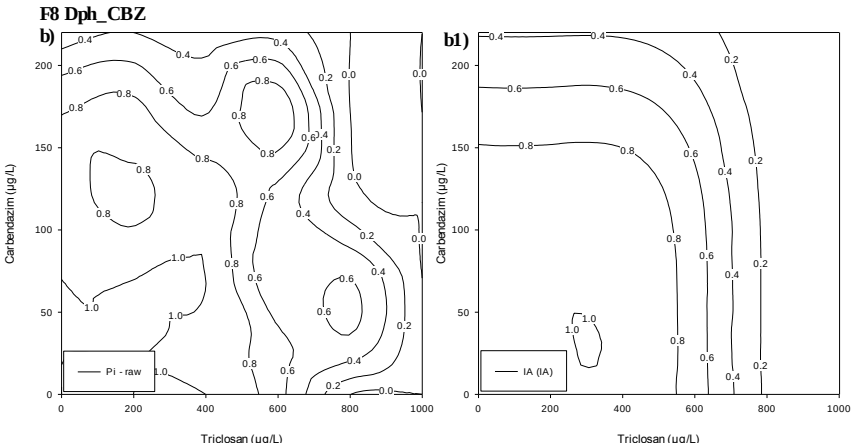
Table 4.2: MIXTOX analysis of the combinations of triclosan and carbendazim to the immobilisation data on the F8 and F12 generations of *Daphnia magna* for Dph_Clean and Dph_CBZ populations using the IA model.

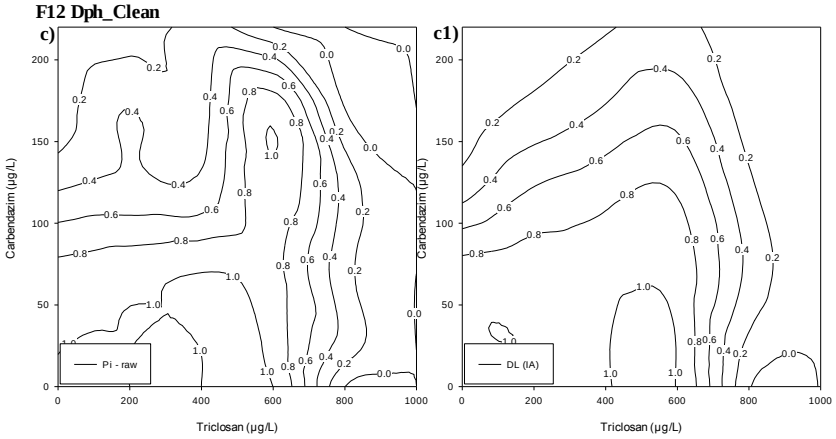
	F8 Immobilisation							
	Dph_Clean Independent Action				**Dph_CBZ** Independent Action			
	IA	S/A	DR	DL	IA	S/A	DR	DL
r^2	0.67	0.73	0.73	0.73	0.84	0.84	0.84	0.85
SS	159.87	130.08	128.10	128.27	70.92	70.90	70.55	68.78
***p*(*F*-test)**	**$3.6x10^{-68}$**	-	-	-	**$8.73x10^{-81}$**	-	-	-
***p*(χ^2)**	-	**$4.8x10^{-8}$**	0.15	0.17	-	0.91	0.83	0.34
max	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.98
a	-	4.56	6.97	8.16	-	-0.049	-0.94	-1.95
b	-	-	-4.97	0.65	-	-	1.70	1.75

	F12 Immobilisation							
	Dph_Clean Independent Action				**Dph_CBZ** Independent Action			
	IA	S/A	DR	DL	IA	S/A	DR	DL
r^2	0.84	0.85	0.89	0.90	0.78	0.84	0.84	0.86
SS	82.42	76.42	53.66	52.13	89.07	65.08	64.40	57.83
***p*(*F*-test)**	**$1.90x10^{-90}$**	-	-	-	**$9.15x10^{-67}$**	-	-	-
***p*(χ^2)**	-	**0.014**	**$1.8x10^{-6}$**	**$8.26x10^{-7}$**	-	**$9.7x10^{-7}$**	0.41	**0.0071**
max	0.98	0.98	0.98	0.98	0.94	0.93	0.92	0.95
a	-	1.15	0.51	0.036	-	6.02	10.13	0.15
b	-	-	16.16	-213.57	-	-	-7.41	-63.37

r^2 represents the maximum likelihood test; SS represents the sum of squared residuals; *p*(*F*-test) represents the result of the likelihood ratio test; *p*(χ^2) represents the outcome of the likelihood ratio test; max represents the control response, *a* and *b* represents the additional parameters of the function; IA represents the independent action model; S/A represents synergism/antagonism; DR represents the dose ratio dependence; DL represents the dose level dependence.


F8 Dph_Clean
a)
a1)
Carbendazim (µg/L)
Triclosan (µg/L)
Pi - raw
A (IA)


F8 Dph_CBZ
b)
b1)
Carbendazim (µg/L)
Triclosan (µg/L)
Pi - raw
IA (IA)


F12 Dph_Clean
c)
c1)
Carbendazim (µg/L)
Triclosan (µg/L)
Pi - raw
DL (IA)

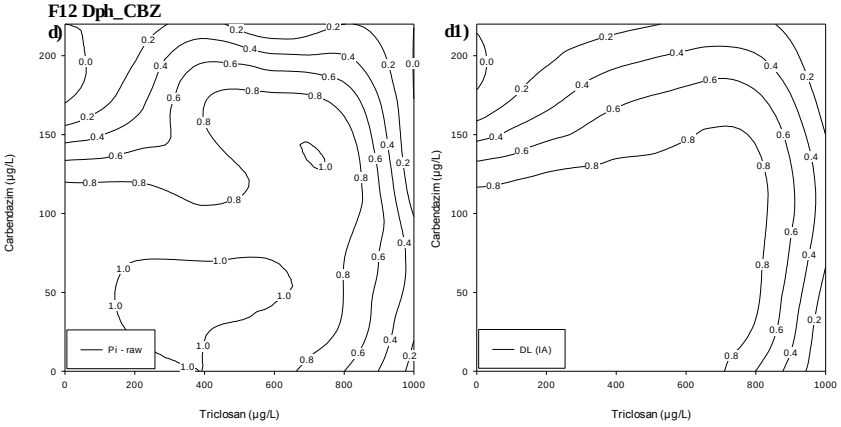

F12 Dph_CBZ
d)
d1)
Carbendazim (µg/L)
Triclosan (µg/L)
Pi - raw
DL (IA)

Figure 4.4. Concentration-response relationship for the binary mixture of triclosan and carbendazim for the immobilisation data in the F8 and F12 generations of *Daphnia magna* (2D isobolic surfaces)): **F8 Dph_Clean a)** observed data, **a1)** modelled data showing antagonistic; **F8 Dph_CBZ b)** observed data, **b1)** IA modelled data; **F12 Dph_Clean c)** observed data, **c1)** modelled data showing a Dose Level (DL) deviation; **F12 Dph_CBZ d)** observed data, **d1)** modelled data showing a Dose Level (DL) deviation.

Assumptions on what might have caused this difference in the mixture toxicity patterns in F8 Dph_CBZ (IA while other is presenting antagonism) are difficult to carry out. Randomness can be a plausible explanation. Looking at the isobologram from Fig. 4.4b and 4.4b1, the raw data and the modelled data (respectively) show curves bending to antagonism. Although the IA model was not statistically improved extending the equation with parameter *a*, a potential decrease on the joint toxicity can be assumed when comparing the observed data with the predicted data.

In addition, patterns derived from the CA model for the binary mixture with TCS and CBZ towards antagonism also support similar findings and are presented in the supplementary data (Table 4.1 SD and Fig. 4.2 SD). Differences in the toxicity prediction between both conceptual models are small and distinctions regarding MoA used to decide which reference model to choose is hard (Cedergreen *et al.*, 2008; EFSA, 2015).

3.4 Pulse and mixture exposure: Comet assay

The comet assay was performed in the present work with the purpose of evaluating the genotoxic effects of TCS, CBZ and its binary mixture in daphnids previously exposed to CBZ during several generations.

In the F12 generation, the percentage of DNA damage increased with increasing concentrations of CBZ and TCS for both Dph_Clean and Dph_CBZ populations (Fig. 4.5a and 4.5b).

After exposure to CBZ, significant effects of the compound were detected for the DNA damage (two-way ANOVA, $F_{3,23}$ = 56.923, $p<0.001$) (Fig. 4.5a and Table 4.3). Significant differences were observed between Dph_Clean and Dph_CBZ populations (two-way ANOVA, $F_{1,23}$ = 44.871, $p<0.001$) (Fig. 4.5a and Table 4.3) and this factor explained 13% of the total variation (Table 4.3). For the Dph_Clean population there was a higher increase on the percentage of DNA damage (with increasing concentrations)

comparing with Dph_CBZ population (Fig. 4.5a). A pre-exposure to CBZ for twelve generations (F12) appeared to have worsened the effect of a new exposure to CBZ at all concentration with the only exception for the last one (Fig. 4.5a). Both factors interacted, indicating that populations responded differently to CBZ concentrations (two-way ANOVA, $F_{3,23} = 34.186$, $p<0.001$).

After exposure to TCS, significant DNA damage was also detected (two-way ANOVA, $F_{3,23} = 30.671$, $p<0.001$) (Table 4.3) and significant differences were observed between Dph_Clean and Dph_CBZ populations (two-way ANOVA, $F_{1,23} = 61.346$, $p<0.001$) (Table 4.3). Similarly to what occurred after exposure to CBZ, Dph_CBZ population presented always a higher percentage of DNA damage compared with Dph_Clean population at all TCS concentrations (Fig. 4.5 b). The population factor explained 35% of the total variation (Table 4.4). However, both factors did not interact, indicating that populations responded similarly to the concentrations of TCS (two-way ANOVA, $F_{3,23} = 2.119$, $p>0.05$), which is well noticed in Fig. 4.5b.

Significant effects on DNA damage were detected upon exposure to the binary mixtures of CBZ and TCS (two-way ANOVA, $F_{3,23} = 40.657$, $p<0.001$) (Fig. 4.5c and Table 4.3). Significant differences were found between Dph_Clean and Dph_CBZ populations (two-way ANOVA, $F_{1,23} = 21.610$, $p<0.001$) (Fig. 4.5c and Table 4.3) and both factors interacted, indicating that populations responded differently to the mixture exposures (two-way ANOVA, $F_{3,23} = 8.463$, $p<0.05$) (Fig. 4.5c). The population factor explained 12% of the total variation (Table 4.3).

Genotoxic effects have been reported in different organisms for both CBZ (in the plant *Hordeum vulgare* L and marine invertebrate *Donax faba*) and TCS (in the zebra mussel *Dreissena polymorpha* and algae *Closterium ehrenbergii*) (Binelli *et al.*, 2009; Ciniglia *et al.*, 2005; JanakiDevi *et al.*, 2013; Singh *et al.*, 2008). Nevertheless, these genotoxic studies usually disregard a possible pre-exposure that might occur. Noteworthy is that, in the present work, genotoxicity (as DNA damage) was generally higher in organisms that were exposed to CBZ (Dph_CBZ) for twelve generations (F12), compared with organisms that were always kept in clean medium (Dph_Clean). This occurred after the exposure to CBZ, TCS and mixture of both (at almost all concentrations and combinations) (Fig. 4.5 a, b and c). One hypothesis for this increase could be the

transmission of DNA damage from parents to neonates throughout the generations. A possible accumulation and transmission of DNA damage to the offspring was demonstrated in a generation experiment with *D. magna* and benzo(a)pyrene (Atienzar and Jha, 2004) and also in daphnids exposed to depleted uranium (Plaire *et al.*, 2013). Additionally, genotoxicity is dependent on the efficiency of several repair mechanisms (Jha, 2008) and some chemicals might affect those mechanisms of repair, making them less effective (reduced or slower), contributing to an increase in DNA damage (Collins *et al.*, 1995).

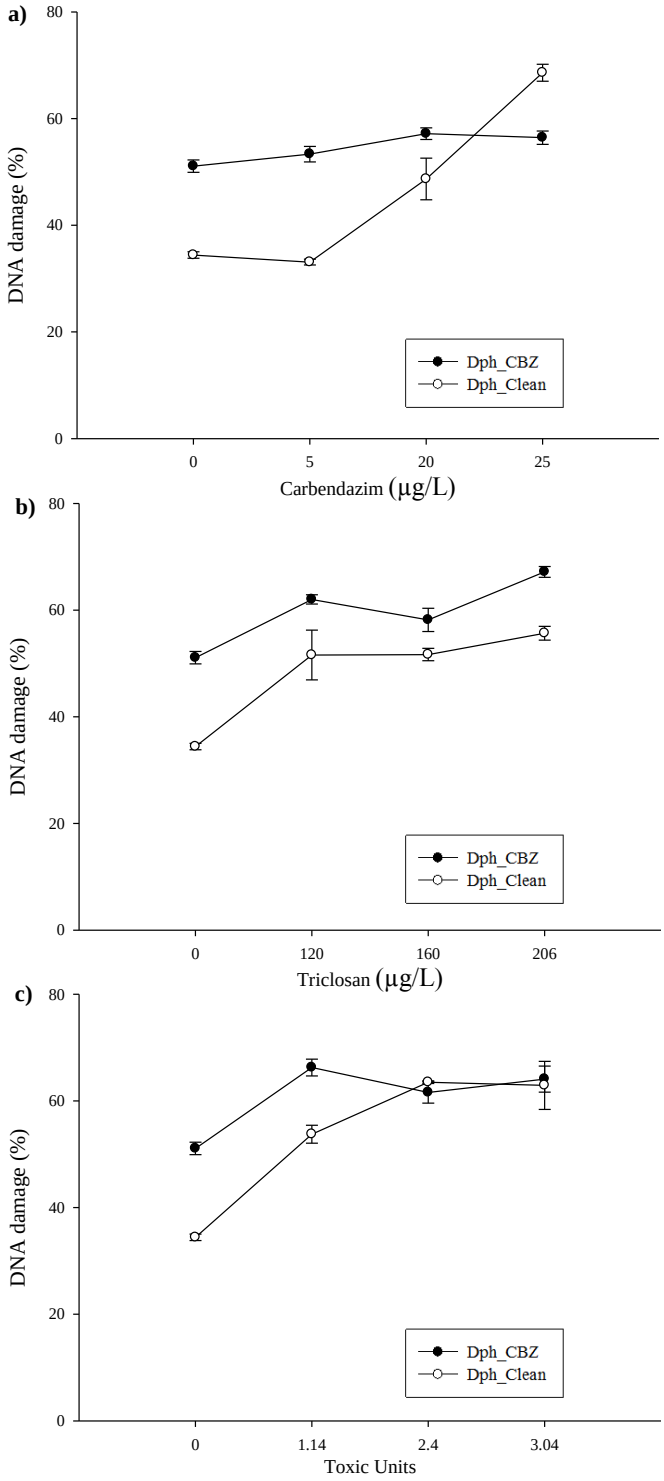


Figure 4.5. DNA damage (%) in F12 *Daphnia magna* cells from populations kept in clean medium- Dph_Clean (white dots), and from populations kept in CBZ- Dph_CBZ (black dots) during the multigenerational test: **a)** exposure to carbendazim **b)** exposure to triclosan **c)** exposure to the mixture of carbendazim and triclosan (Toxic Units). Data are expressed as mean values and standard error.

Table 4.3. Two-way ANOVA results testing for effects of exposure/populations of *Daphnia magna* (Dph_Clean *vs.* Dph_CBZ) after carbendazim exposure ([CBZ]), triclosan ([TCS]) or mixture with carbendazim and triclosan ([MIXT]) and their interaction on the DNA damage in the F12 generation.

	DF	Sum of squares	F	*p*-value	R^2
F12 DNA damage after carbendazim exposure					
[CBZ]	3	1577.841	56.923	**<0.001**	0.51
Population	1	414.586	44.871	**<0.001**	0.13
[CBZ] x Population	3	947.591	34.186	**<0.001**	0.31
F12 DNA damage after triclosan exposure					
[TCS]	3	1141.195	30.671	**<0.001**	0.52
Population	1	762.190	61.346	**<0.001**	0.35
[TCS] x Population	3	78.987	2.119	0.138	0.04
F12 DNA damage after mixture exposure					
[MIXT]	3	1709.195	40.657	**<0.001**	0.66
Population	1	302.815	21.610	**<0.001**	0.12
[MIXT] x Population	3	355.779	8.463	**0.001**	0.14

When DNA damage was assessed in the Dph_CBZ population upon TCS exposure (Fig. 4.5b, Dph_CBZ), daphnids at the control (0 μg TCS/L) are depicting DNA damage from the multigenerational exposure. Therefore, by comparing this response upon no CBZ exposure with the increasing TCS concentrations, one can observe that the pulse exposure to TCS decreased the fitness of individuals, by increasing the DNA damage (Dunnett's Method, $p<0.05$). This was also observed for the mixture exposure (Fig. 4.5c, Dph_CBZ).

For the DNA damage, CBZ and TCS did not induce a full dose-response relationship. For this reason, the EC_{50} values were underestimated and further analysis in the MIXTOX tool was undertaken with fixed EC_{50} values. This alternative was already implemented with success in the study by Loureiro *et al.* (2010) and Pérez *et al.* (2013). For the F12 generation, the IA model fitted the data significantly for Dph_Clean population. When adding parameters for deviations, the DR deviation fitted the data significantly, a negative *a* value and a positive *b* value (Table 4.4 and Fig. 4.6a1). This indicates that synergism ($a<0$) was mainly caused by TCS, except for mixture ratios where antagonism is observed and caused mainly by CBZ ($b>0$) (Table 4.1). For the F12 generation for Dph_CBZ population, data was significantly adjusted to the IA model. Extending the model with parameters *a* and b_{DR}, a significant decrease in the SS value was observed, showing the best fit to the data (Table 4.4). Similar to the pattern in the F12

Dph_Clean, the parameter *a* was negative and parameter *b* was positive, meaning that synergism was mainly caused by TCS (Table 4.4 and Fig. 4.6b1).

It should be noted that, in the aquatic environment these two compounds might appear together and that synergism (predicted in the DR deviation) was observed in both Dph_Clean and Dph_CBZ populations.

Considering the above mentioned, patterns derived from the CA model for the binary mixture with TCS and CBZ for DNA damage are presented in the supplementary data as well and support generally similar findings (Table 4.1 SD and Fig 4.3 SD).

Table 4.4: MIXTOX analysis of the combinations of triclosan and carbendazim to the % of no DNA damage on *Daphnia magna* cells for Dph_Clean and Dph_CBZ populations, on the F12 generation.

	F12 % of no DNA damage							
	Dph_Clean Independent Action				**Dph_CBZ** Independent Action			
	IA	S/A	DR	DL	IA	S/A	DR	DL
r^2	0.74	0.77	0.84	0.83	0.57	0.66	0.75	0.68
SS	1069.91	942.8	668.76	716.31	412.03	320.60	237.66	304.88
***p*(*F*-test)**	**4.80×10^{-7}**	-	-	-	**0.0002**	-	-	-
$p(\chi^2)$	-	0.051	**0.00087**	**0.0024**	-	**0.0061**	**0.0027**	0.22
max	66.14	66.13	67.03	65.81	46.57	50.40	48.86	50.29
a	-	1.18	-7.05	0.0023	-	5.67	-21.37	0.22
b	-	-	11.60	-1517.31	-	-	379.16	-632.39

r^2 represents the maximum likelihood test; SS represents the sum of squared residuals; *p*(*F*-test) represents the result of the likelihood ratio test; $p(\chi^2)$ represents the outcome of the likelihood ratio test; max represents the control response, *a* and *b* represents the additional parameters of the function; IA represents the independent action model; S/A represents synergism/antagonism; DR represents the dose ratio dependence; DL represents the dose level dependence.

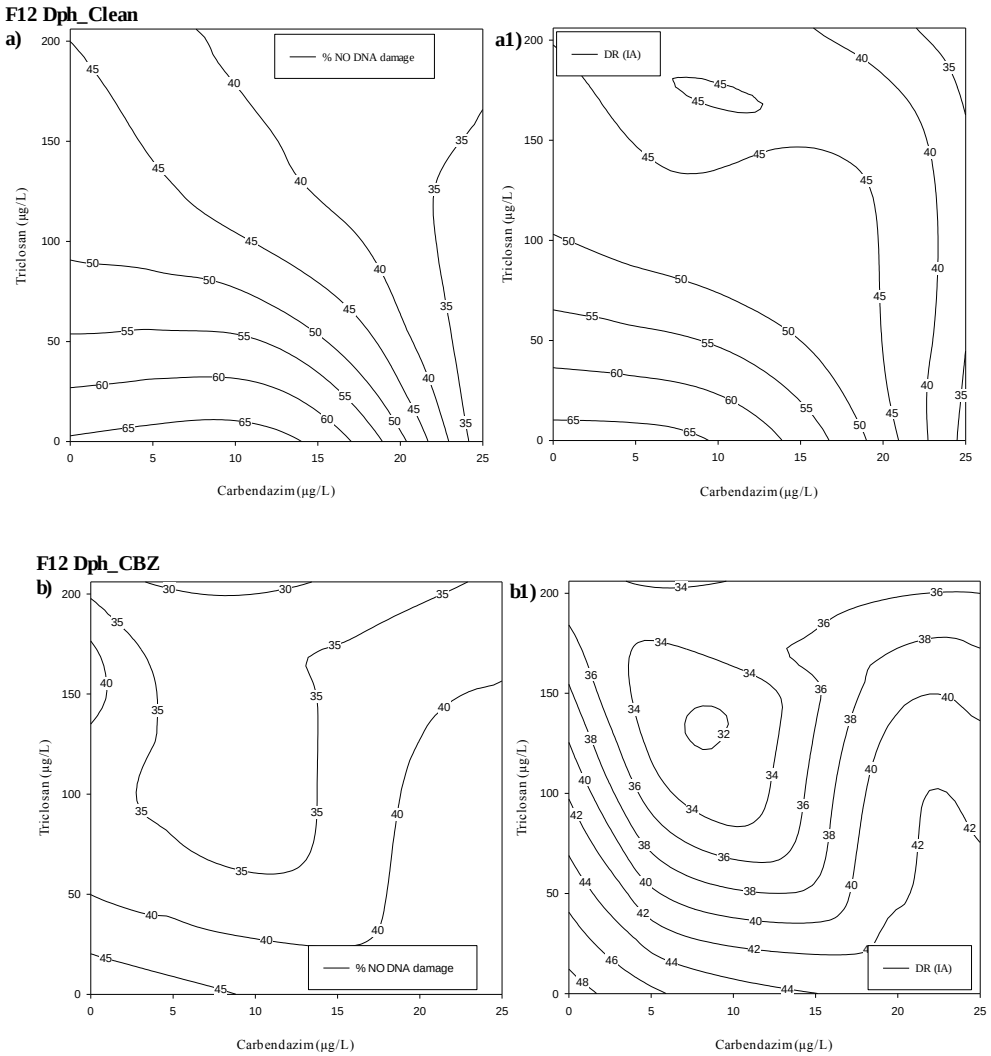


Figure 4.6. Concentration-response relationship for the binary mixture of triclosan on the % of no DNA damage on *Daphnia magna* cells (2D isobolic surfaces): **F12 Dph_Clean a)** observed data **a1)** modelled data showing a Dose Ratio (DR) deviation; **F12 Dph_CBZ b)** observed data **b1)** modelled data showing a Dose Ratio (DR) deviation.

Similar mixture patterns for the combination of TCS and CBZ were found previously for *D. magna*: DL dependency with antagonism observed at low doses of the chemical mixture for the immobilisation data and DR dependency with synergism mainly driven by TCS for the DNA damage (Chapter 2 - Silva *et al.*, 2015).

4. Conclusions

The present study demonstrates that the long-term exposure to CBZ, lasting for 12 generations, induced no changes on daphnids' sensitivity to TCS. This conclusion was derived from the similar 48h-LC_{50} values obtained. However, daphnids that were exposed to CBZ for twelve generations presented generally a higher percentage of DNA damage than those maintained always in clean medium, and tested for CBZ, TCS and their binary combination. DNA damage might have consequences in fitness, adaptability and survival of the organisms, consequently affecting the ecosystem quality (Jha 2008). Considering the mixture patterns after the exposure to CBZ and TCS, patterns were generally similar in daphnids in clean medium compared with daphnids exposed to CBZ for the immobilisation and DNA damage data.

The present study can add value towards hazard and risk assessment of CBZ, TCS but also of their mixtures in the environment. The multigenerational effects caused by chemicals highlight the need to develop a standardized protocol and can bridge information from individuals to populations.

Acknowledgments

This work was supported by the project RePulse- Responses of *Daphnia magna* exposed to chemical pulses and mixtures throughout generations (FCOMP-01-0124-FEDER-019321; Refª. FCT PTDC/AAC-AMB/117178/2010), by funding FEDER through COMPETE- Programa Operacional Factores de Competitividade, and by the Portuguese Science Foundation (FCT) through CESAM: UID/AMB/50017/2013. Ana Rita Rego Gouveia Silva was funded by the project "MARPRO- Conservation of marine protected species in Mainland Portugal" through the doctoral fellowship (BD/UI88/5534/2011), Diogo Cardoso was funded by the doctoral grant (PD/BD/52569/2014) and Andreia Cruz was funded by an individual post-doctoral grant (BPD/UI88/2886/2013), within the project "Sustainable Use of Marine Resources" - MARES (CENTRO-07-ST24-FEDER-002033), financed by QREN, Mais Centro- Programa Operacional Regional do Centro e União Europeia/ Fundo Europeu de Desenvolvimento Regional.

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Supplementary data

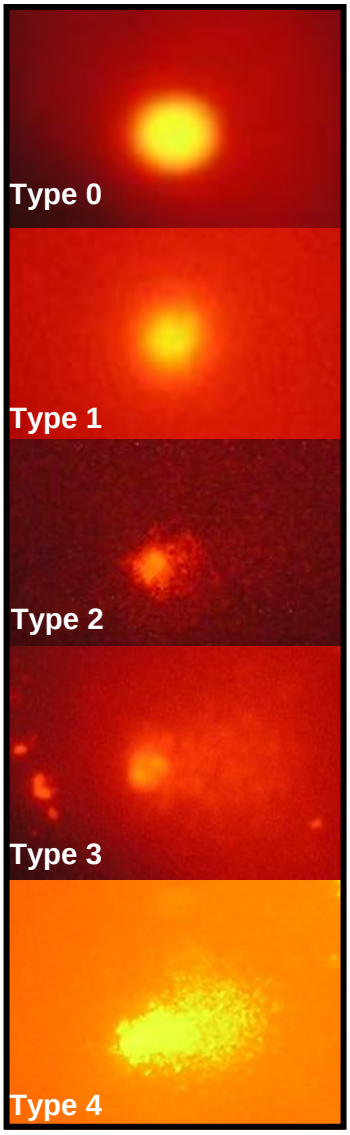


Figure 4.1 SD. Comet type scale used in daphnid cells.

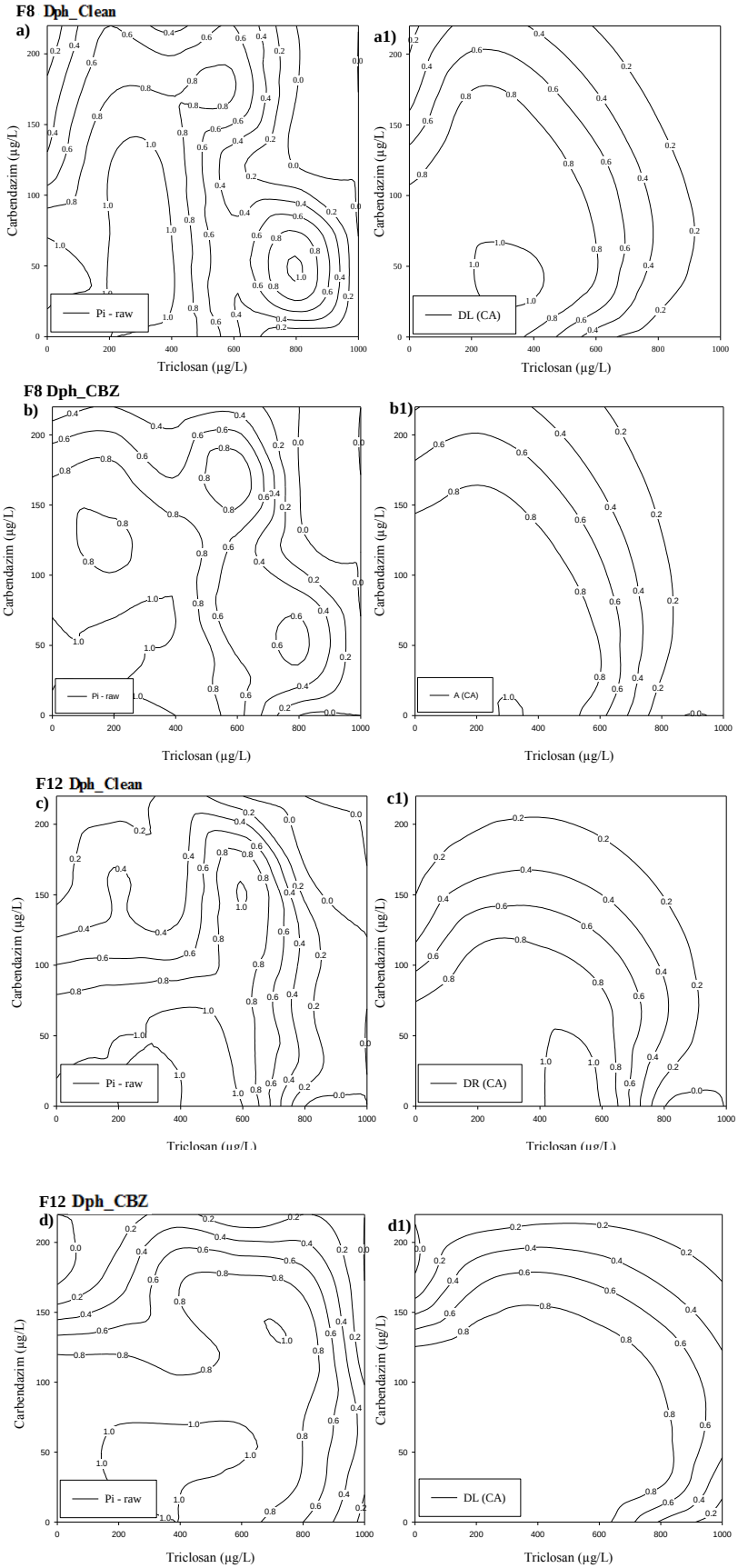


Figure 4.2 SD. Concentration-response relationships for the binary mixture of triclosan and carbendazim on the immobilisation data for the F8 and F12 generations of *Daphnia magna* (2D isobolic surfaces): **F8**

Dph_Clean a) observed data **a1)** modelled data showing a Dose Level (DL) deviation; **F8 Dph_CBZ b)** observed data **b1)** modelled data showing an antagonistic (A) deviation; **F12 Dph_Clean c)** observed data **c1)** modelled data showing a Dose ratio (DR) deviation; **F12 Dph_CBZ d)** observed data **d1)** modelled data showing a Dose Level (DL) deviation.

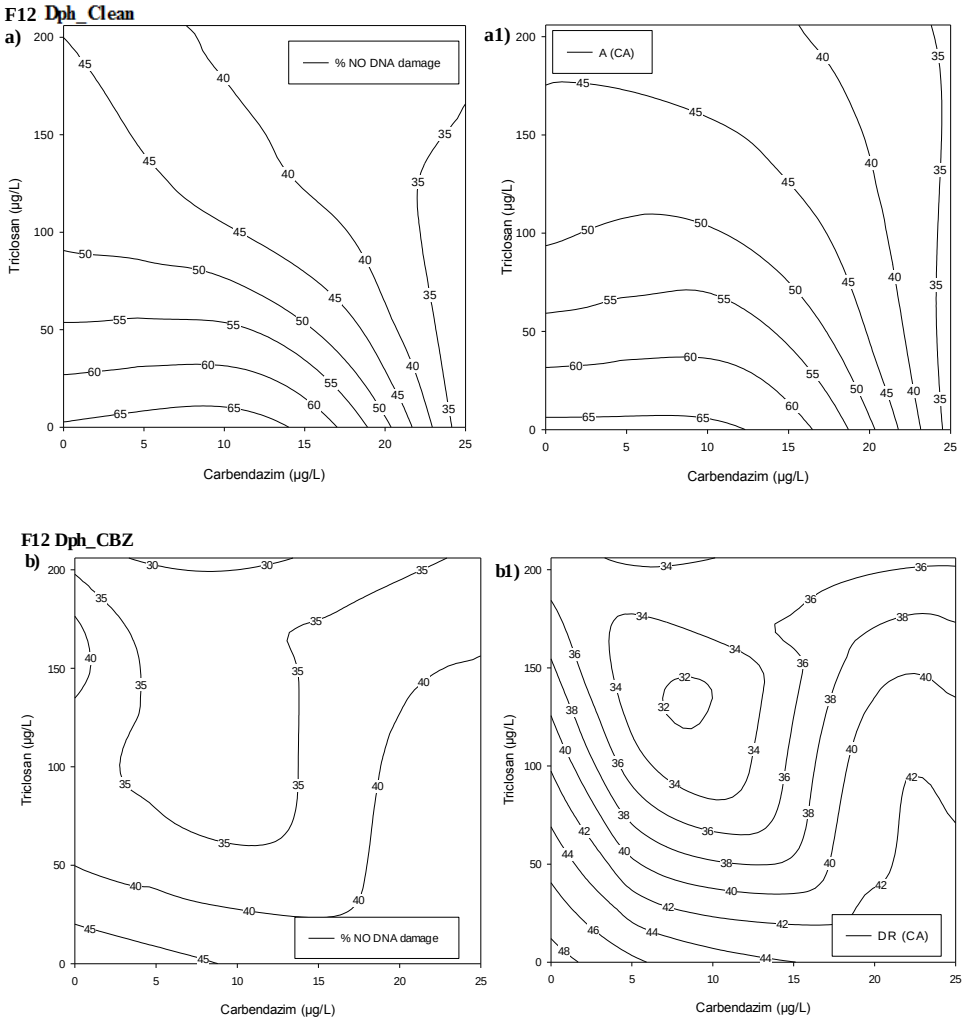


Figure 4.3 SD. Concentration-response relationship for the binary mixture of triclosan and carbendazim on the % of no DNA damage on *Daphnia magna* cells for the F12 generation (2D isobolic surfaces): **F12 Dph_Clean a)** observed data **a1)** modelled data showing an Antagonistic (A) deviation; **F12 Dph_CBZ b)** observed data **b1)** modelled data showing a Dose Ratio (DR) deviation.

Table 4.1 SD. MIXTOX analysis of the combinations of triclosan and carbendazim using the CA model to the **a)** immobilisation data of the F8 and F12 generations of *Daphnia magna* and for the **b)** % of no DNA damage in F12 generation for Dph_Clean and Dph_CBZ populations.

a)

	F8 Immobilisation							
	Dph_Clean Concentration Addition				**Dph_CBZ** Concentration Addition			
	CA	S/A	DR	DL	CA	S/A	DR	DL
r^2	0.54	0.72	0.74	0.73	0.53	0.82	0.83	0.83
SS	220.57	136.17	123.02	128.90	212.23	78.76	78.75	78.67
***p*(*F*-test)**	**$4.47x10^{-55}$**	-	-	-	**$2.67x10^{-50}$**	-	-	-
$p(\chi^2)$	-	**$4x10^{-20}$**	**0.00029**	**0.0070**	-	**$7.1x10^{-31}$**	0.92	0.76
max	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.98
a	-	3.13	4.94	5.78	-	2.02	1.98	2.38
b	-	-	-3.81	0.19	-	-	0.069	0.092

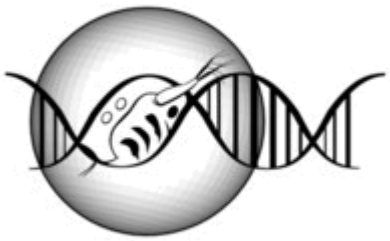
	F12 Immobilisation							
	Dph_Clean Concentration Addition				**Dph_CBZ** Concentration Addition			
	CA	S/A	DR	DL	CA	S/A	DR	DL
r^2	0.62	0.84	0.85	0.84	0.41	0.75	0.83	0.80
SS	194.35	83.81	77.24	83.54	237.84	221.45	67.57	78.86
***p*(*F*-test)**	**$2.82x10^{-66}$**	-	-	-	**$9.76x10^{-35}$**	-	-	-
$p(\chi^2)$	-	**$7.5x10^{-26}$**	**0.010**	0.61	-	**$5.2x10^{-5}$**	**$2.5x10^{-35}$**	**$7.2x10^{-33}$**
max	0.98	0.98	0.97	0.98	0.96	0.96	0.92	0.93
a	-	3.04	4.65	3.28	-	0.80	4.87	2.29
b	-	-	-4.19	0.038	-	-	-3.75	-0.16

b)

	F12 % of no DNA damage							
	Dph_Clean Concentration Addition				**Dph_CBZ** Concentration Addition			
	CA	S/A	DR	DL	CA	S/A	DR	DL
r^2	0.81	0.88	0.88	0.89	0.60	0.63	0.75	0.64
SS	788.88	508.35	480.28	459.69	383.35	347.67	236.92	337.98
***p*(*F*-test)**	**$1.15x10^{-8}$**	-	-	-	**$9.83x10^{-5}$**	-	-	-
$p(\chi^2)$	-	**0.00028**	0.19	0.08	-	0.087	**0.00073**	0.15
max	69.56	66.28	66.04	65.93	49.20	49.07	48.85	49.07
a	-	1.50	-4.74	0.012	-	4.46	-39.12	0.013
b	-	-	8.07	-125.7	-	-	628.89	-814.66

r^2 represents the maximum likelihood test; SS represents the sum of squared residuals; p(*F*-test) represents the result of the likelihood ratio test; $p(\chi^2)$ represents the outcome of the likelihood ratio test; max represents the control response, *a* and *b* represents the additional parameters of the function; CA represents the concentration addition model; S/A represents synergism/antagonism; DR represents the dose ratio dependence; DL represents the dose level dependence.

Chapter 5



From mother to offspring: a multigenerational study on the individual and subcellular level effect of carbendazim on Daphnia magna

From mother to offspring: a multigenerational study on the individual and subcellular level effect of carbendazim on *Daphnia magna*

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Abstract

Anthropogenic factors such as the use of pesticides may have disastrous consequences to aquatic populations. Carbendazim is one example of a widely used fungicide with a high potential to end up in aquatic ecosystems. The deleterious effects observed at the population level can often be depicted or explained by changes in homeostasis at cellular and individual levels. In the present study, an isoclonal population of *Daphnia magna* (clone k6) was exposed to an environmentally relevant concentration (5 µg/L) of carbendazim during thirteen generations. The effects of carbendazim on survival/longevity, reproduction, length of mothers, DNA damage (determined by comet assay), biochemical biomarkers (cholinesterase, catalase and glutathione *S*-transferase), lipid peroxidation and energy-related parameters (carbohydrates, lipids and proteins along with energy available and energy consumption) were assessed at some generations. The results showed that a long-term exposure to carbendazim had no effect on the intrinsic rate of natural increase (*r*) and length of *D. magna.* However, daphnids exposed to carbendazim, showed a decrease in longevity in the F12 generation along with an increase in DNA damage from generation F3 to F13 when compared with the isoclonal population in clean medium. Cholinesterases, glutathione *S*-transferase and lipid peroxidation showed differences between the population exposed to carbendazim and the population in clean medium. However, for catalase and energy related-parameters, no differences were observed between these two populations. Overall, no clear pattern regarding changes in sensitivity was observed in daphnids exposed to carbendazim throughout generations.

Key words: *Daphnia magna*, multigenerations, carbendazim, biochemical biomarkers, energy reserves, DNA damage

1. Introduction

Over the last decades, pesticides have been extensively used in agriculture across the globe (Ecobichon, 2001). Due to the possible continuous use of these compounds and consequently their inevitable presence in aquatic systems, organisms may be exposed throughout numerous generations and, therefore, assessing multigenerational effects is thus of utmost relevance. Although some multigenerational studies have already been carried out with pesticides (Brausch and Smith, 2009; Liess *et al.*, 2013), no clear conclusions could be drawn regarding long-term effects at the population level.

The possibility to work with clonal lineages and generate genetically identical offspring, makes the water flea *Daphnia magna* a good species to test effects on multigenerations due to its parthenogenetic reproduction (Hebert and Ward, 1972). Therefore, population studies can be simulated at the laboratorial scale in order to predict effects at this higher organizational level.

Although environmental concentrations of toxic compounds are generally low with no inherent acute toxicity observed, such conditions can still cause sublethal effects that reduce organisms' fitness. This might be related, for instance, with the accumulation of damage at a sub-organismal level, such as DNA damage, changes in enzymatic pathways and unbalanced internal energy budget, thus affecting general endpoints (*e.g.* growth or reproduction) (De Coen and Janssen, 2003b). Studying the effects at a subcellular level is an important tool in toxicology, as it will improve the knowledge on the chemical modes of action allowing to establish a linkage between effects at the cellular and organismal levels.

Biomarkers can be considered measures of initial changes, in organisms, in response to toxic compounds. Accordingly, the following biomarkers were selected: Cholinesterases (ChE) activity, a well-known target site of carbamate pesticides, which inhibits its activity triggering neurotoxic effects in *D. magna* (Barata *et al.*, 2004); catalase (CAT) as an antioxidant enzyme (Brown *et al.*, 2004); glutathione *S*-transferases (GST) is related with biotransformation and antioxidant defense (Hyne and Maher, 2003); and finally lipid peroxidation (LPO) rate, which is associated with cell damage (Barata *et al.*, 2005). These indicators can provide more information regarding changes in sensitivity upon a long-term

exposure of a population, thus helping to better understand the modes of action of chemical compounds and also relate it to effects at higher levels of organization.

Carbendazim (CBZ) (methyl-2-benzimidazole carbamate) has been used for many years as a fungicide in several crops, including potatoes, strawberries, onions, wheat, oranges, among others (EU Pesticide Database, 2015) and consequently it is likely to be continuous released from spring to autumn. After its application, it may pass into aquatic systems due to spray-drift or run-off from crops and soils (after rainfall events) (WHO, 1993). CBZ is persistent in the water layer (Cuppen *et al.*, 2000), and it was found in concentrations near 5 µg/L in surface waters (Palma *et al.*, 2004). CBZ have already been reported to cause subcellular effects in several species, including genotoxicity (JanakiDevi *et al.*, 2013; Chapter 2 - Silva *et al.*, 2015; Singh *et al.*, 2008). Therefore, in the present study, it will be used as a model chemical. In addition, results from a previous work showed that DNA damage (genotoxicity) increased throughout generations in a multigenerational test with *D. magna* exposed to CBZ (Chapter 3).

The aims of the present study were to assess the sublethal effects of a long-term exposure to CBZ over thirteen generations of *D. magna,* by evaluating (*i*) the effects on the intrinsic rate of natural increase (*r*), length and longevity of *D. magna* and link them to (*ii*) the effects occurring at a subcellular level, using biochemical indicators ChE, CAT and GST activities, and LPO rate, energy-related parameters (lipids, carbohydrates and proteins content, energy consumption and energy available) and DNA damage (using the comet assay).

2. Materials and methods

2.1 Test organism

The water flea *D. magna* Straus clone K6 (originally from Antwerp, Belgium) was obtained from continuous culture maintained in a laboratory at the University of Aveiro (Portugal) and cultured in American Society for Testing and Materials moderated-hard-water medium (ASTM, 1980), with a temperature 20 ± 1^{0}C and a 16h:8h (light:dark) photoperiod. The medium was renewed three times a week and daphnids were fed with the microalga *Raphidocelis subcapitata* (formerly known as *Pseudokirchneriella subcapitata*)

at a concentration of $3x10^5$ cells/mL and supplemented with an organic extract (Marinure seaweed extract, supplied by Glenside Organics Ltd.).

2.2 Test chemicals

A stock solution of carbendazim (CAS No. 10605-21-7, 99.4% purity, Bayer Crop Science) was prepared in ASTM medium and used to maintain the multigenerational test. Chemical analyses were performed to confirm concentrations of CBZ in the test medium at Marchwood Scientific Services, Southampton, UK. CBZ was analysed by Liquid Chromatography-Mass Spectrometry (LCMS-MS) using the Querchers method. A representative portion of the sample (200-300 mL) was extracted with 20 mL of acetonitrile (containing 1% acetic acid). This was followed by a partitioning step with magnesium sulphate and a subsequent buffering step with sodium acetate. After mixing an aliquot with methanol, the extract was injected directly into the LCMS-MS system (instrument Agilent 6410 Triple Quad LCMS-MS) without any clean-up. A 10 µl injection volume was utilized. Standards were prepared in solvents at seven levels with recoveries in the range 70-120%. Chemical analysis data were then included in a decay model, to assess the degradation constant in time, using the following equation:

(Equation 1) $C_t = C_0 e^{-k0t}$

Where C_0 corresponds to the initial external concentration (µg/L), K_0 corresponds to the constant of degradation of the chemical in the medium (/hour) and t corresponds to time (hours) (Widianarko and Van Straalen, 1996).

2.3 Multigenerational experimental setup

Preliminary toxicity tests were conducted to determine the toxicity of CBZ to *D. magna* using several endpoints (Chapter 2 - Silva *et al.*, 2015). Afterwards, and based on the results of the reproduction test, daphnids isoclonal populations were exposed throughout different generations to the equivalent no-observed-effect-concentration (NOEC) value of the preliminary reproduction test, corresponding to 5 µg/L of CBZ (Chapter 2 - Silva *et al.*, 2015). The experimental design of the multigenerational experiment is shown in Figure 5.1 and will be further described in the following subtopics.

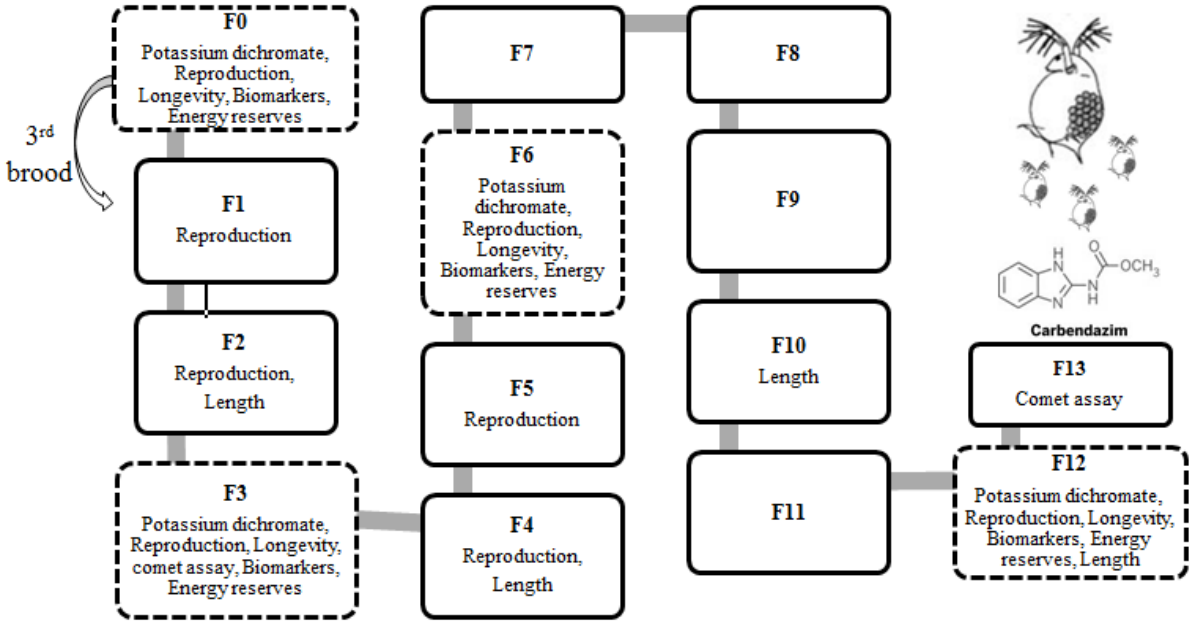

Figure 5.1. Experimental design of the multigenerational experiment. Each box represents a generation and the respective endpoints evaluated or bioassays carried out.

During the multigenerational experiment, an isoclonal population of *D. magna* was continuously exposed to 5 µg/L of CBZ throughout 13 generations. Simultaneously, a second isoclonal population of daphnids was maintained under clean medium and used as control. The population of daphnids that was maintained in a control/clean condition (ASTM, *R. subcapitata* and organic extract but no CBZ) will be designated throughout the study as Dph_Clean and population of daphnids that was exposed to CBZ (ASTM, *R. subcapitata*, organic extract and CBZ) throughout the generations designated as Dph_CBZ.

D. magna cultures consisted of 1L glass vessels containing 1L of culture medium and 20 daphnids, for both populations. Three replicates were used for Dph_Clean and Dph_CBZ, each one consisting in ASTM medium with *R. subcapitata* (concentration of $3x10^5$ cells/mL) and organic extract (Marinure seaweed extract, supplied by Glenside Organics Ltd.) and CBZ in Dph_CBZ. The medium was completely renewed three times a week. Each subsequent generation was always initiated by using third brood neonates (<24h) of the previous one and maintained in the same condition (either Dph_Clean or Dph_CBZ).

In order to control differences in daphnids' responses inherent to sensitivity variations in organisms (Loureiro *et al.*, 2010) all endpoints, reproduction, length, survival, DNA damage, enzyme activities and energy-related parameters were simultaneously

assessed in neonates originating from Dph_Clean and Dph_CBZ throughout the generations.

Sensitivity tests with potassium dichromate (K2Cr2O7) were performed in the F0, F3, F6 and F12 generations to evaluate daphnids sensitivity, according to the OECD procedure (OECD, 2004).

2.3.1 Reproduction and growth

The total number of neonates till the fifth brood was recorded for the Dph_Clean and Dph_CBZ populations from F0 to F6 and also in the F12 generation. These generations were chosen to follow continuously the reproduction in six generations (from F0 to F6) and to provide information for a longer term at F12. The intrinsic rate of natural increase (*r*) was calculated for the same generations where reproduction (as number of neonates) was assessed, using the Euler Lotka equation (Lokta, 1913):

(Equation 2) $\sum_{x=0}^{x} lx\ mx\ e^{-rx} = 1$

where *lx* is the proportion of individuals surviving to age *x*, *mx* is per-capita fecundity, and *x* is days.

The body length (in mm, excluding the antennas and anal spine) was measured in adult daphnids after the fifth brood (21 days old daphnids) in the F2, F4, F10 and F12 generations. These measurements were performed under a stereomicroscope (MS5, Leica Microsystems, Houston, USA).

2.3.2 Longevity

In the F0, F3, F6 and F12 generations, longevity (lifespan in days) was assessed in both Dph_Clean and Dph_CBZ isoclonal populations (for all the 3 replicates of each population). The number of live and dead daphnids was counted over time. A time-response relationship, using the 50% lethal time (LT_{50}) values, was determined.

2.3.3 Comet assay

A pool of *D. magna* neonates was collected from both Dph_Clean and Dph_CBZ populations in F3 and F13 generations to assess DNA damage using the comet assay. The

methodology employed was adapted from Nogueira *et al.* (2006). Four replicates with fifteen juveniles each (<48h) were used. The comet assay was conducted under yellow light to prevent DNA damage. Positive controls consisted of daphnid cells exposed to hydrogen peroxide (H_2O_2). Organisms were placed in 1.5 mL *Eppendorfs* containing 1 mL of phosphate-buffered saline (PBS), 10% dimethyl sulfoxide (DMSO) and 20 µM ethylenediamine tetra-acetic acid (EDTA) and were gently mechanically disintegrated with an appropriate pestle. Samples were centrifuged (200 *g*) during 10 min at 4°C and most of the supernatant was gently removed. 10 µL of the resulting pellet (containing cells), were transferred to *Eppendorfs* containing 0.5% low melting point agarose (at 37°C) and spread on the microscope glass slides pre-coated with 1% normal melting agarose. Then, the slides were placed on ice for 10 min and placed into a lysis solution (10 mM Tris-HCl, 100 mM EDTA, 2.5 M NaCl, 10% DMSO and 10% Triton X-100, pH 10) for 1h to allow cell lysis. Afterwards, slides were placed in the electrophoresis tank, containing the electrophoresis buffer (10 M NaOH, 200 mM Na_2-EDTA, pH 10) and left for 15 min before starting the procedure in order to allow the DNA denaturation (and unwinding). The electrophoresis was run for 10 min by applying an electric current of 300 mA (30 Volts). Thereafter, the slides were washed with 0.4 M Tris-HCl (pH 7.5, to neutralize) and dehydrated with absolute ethanol for 10 sec and left to dry for 1 day in the dark.

For the image analysis, the slides were stained with 100 µL ethidium bromide (20 µL/mL), overlaid with a coverslip visualized under a fluorescence microscope (Olympus BX41TF, China) and observed with 400x magnification. Visual scoring of DNA damage (as comets) was examined in one hundred cells per slide, and each cell was graded on a 0 to 4 scale, as described by Duthie and Collins (1997). Type 0 represents no DNA damage, type 1 and 2 represent mild to moderate damage, respectively, and type 3 and 4 represent extensive DNA damage. The total comet score was calculated according to the same method (Duthie and Collins, 1997): (number of cells in type 0 × (type) 0) + (number of cells in type 1 × (type) 1) + (number of cells in type 2 × (type) 2) + (number of cells in type 3 × (type) 3) + (number of cells in type 4 × (type) 4). Therefore, the total score for 100 cells could range between 0 (all comets with no damage) to 400 (all comets with maximal damage). To ensure unbiased scorings, the scorer was unaware of the treatment condition while reading the slides. A percentage of DNA damage was then calculated. Figure 5.1 SD shows a comet type scale in daphnids' cells.

2.3.4 Biochemical biomarkers' determination

2.3.4.1 Sampling and post-mitochondrial supernatant

For all enzyme analyses, five replicates with sixteen organisms each (7 days old) were used from both Dph_Clean and Dph_CBZ. Organisms were collected and shock frozen in liquid nitrogen and then stored at -80ºC until analyses. Enzymatic activities were measured in F0, F3, F6 and F12 daphnids, and samples prepared for analysis using an adapted protocol described by Ferreira *et al.* (2010). Briefly, daphnids were sonicated (Kika Labortechnik U2005 Control^{TM}) for approximately 3 sec with 1 mL of potassium phosphate buffer 0.1 M (pH 7.4). After sonication, 150 µL of the homogenate was separated to a 2 mL *Eppendorf* containing 2.5 µL butylated hydroxytoluene (BHT) 4% in methanol and used for LPO determination. The remaining homogenate was centrifuged at 10 000 *g* for 20 min at 4 ºC and used for ChE, CAT and GST analyses. Biochemical measurements were then carried out in the resulting supernatant using a LabSystem Multiskan EX microplate reader (Labsystems Inc., Franklin, MA, USA).

2.3.4.2 Protein determination

Protein concentration to be used as correction factor for the chemical biomakers was quantified in quadruplicate in supernatant using the Bradford method (Bradford, 1976), adapted to microplate, using a standard solution of bovine *γ*-globulin, at 595 nm. The sample supernatants were then adjusted to 0.20-0.30 mg protein/mL (ChE) and 0.50-0.80 mg protein/mL (CAT and GST) for posterior enzymatic analysis. All dillutions were done using the respective buffer for each specific biomarker. Final protein concentration was always determined for confirmation.

2.3.4.3 Enzymatic determinations

ChE activity was measured according to the Ellman method (Ellman *et al.*, 1961) adapted to a 96 well microplate as described in Guilhermino *et al.* (1996). Briefly, a reaction solution constituted of 1 mL of 5.50-dithiobis-2-nitrobenzoicacid (DTNB) 10 mM solution, 1.20 mL of 0.075 M acetylthiocholine iodide solution and 28.920 mL of 0.1 M phosphate buffer was used and added to 50 µL of the sample; after 10, 15 and 20 min the

absorbance at 414 nm was read. The enzymatic activity was then calculated using the coefficient of extinction (ε=1.36 x $10^3 M^{-1}$ cm^{-1}) and expressed as nanomol (nmol) of substance hydrolysed per min.

CAT activity was determined based on the methodology described by Claiborne (1985) adapted to microplate (Ferreira *et al.*, 2015). In short, 135 µL K-phosphate 0.05 M (pH 7.0) and 150 µL H_2O_2 0.03 M was added to 15 µL of the sample supernatant, shortly before absorbance was measured at 240 nm for 1 min. The enzymatic activity was calculated regarding the decomposition of the substrate H_2O_2 by using the coefficient of extinction (ε= 40 M^{-1} cm^{-1}). The enzymatic activity was expressed as µmol of substrate hydrolyzed per min.

GST activity was determined according to the method described by Habig *et al.* (1974) adapted to microplate (Frasco and Guilhermino, 2002). For that, 100 µL of the sample was mixed with 200 µL of the reaction solution (29.70 mL K-phosphate buffer 0.1 M (pH 6.5), 0.9 mL 1-chloro-2.4-dinitrobenzene (CDNB) 10 mM and 5.4 mL $_L$-glutathione reduced (GSH - 10 mM), and the absorbance read at 340 nm. The enzymatic activity was expressed as nanomol (nmol) of substrate hydrolyzed per min using a coefficient of extinction (ε= 9.6 x 10^3 M^{-1} cm^{-1}).

2.3.4.4 Lipid peroxidation determination

LPO was determined as described by Ohkawa *et al.* (1979) and Bird and Draper (1984), adapted to microplate, by measuring the production of thiobarbituric acid-reactive substances (TBARS) at 535 nm. In brief, to each 150 µL of the homogenate aliquot, 500 µL trichloro acetic acid sodium salt (TCA) 12% (w/v), 400 µL Tris-HCl 60 mM with diethylenetriamine penta acetic acid (DTPA) and 500 µL 2-thiobarbituric acid (TBA) 0.73% (w/v) were added. *Eppendorfs* were then incubated at 100ºC for 1h and after that, centrifuged at 11 500 *g* for 5 min at 25ºC. Samples were protected from light during placement in the microplate and before absorbance measurements at 535 nm. LPO was expressed as nanomols of TBARS formed per mg of wet weight, using the extinction coefficient (ε= 1.56 x 10^5 M^{-1} cm^{-1}).

2.3.5 Energy reserves determination

For measuring the energy reserves, three replicates with twenty daphnids (7 days old) were used. Organisms were collected, shock frozen with liquid nitrogen and stored at -80ºC until further analyses. Energy reserves were measured in F0, F3, F6 and F12 daphnids using a protocol adapted from Ferreira *et al.* (2015) previously described by DeCoen and Janssen (1997). Daphnids were homogenized in 1 mL of distilled water using a sonicator (Kika Labortechnik U2005 Control™), and divided into three 2 mL *Eppendorfs* each one containing 300 µL of the homogenate. One part was used to determine the proteins and carbohydrates contents, the other one to determine the lipids content and the final one to determine the energy consumption (Ec), as electron transport activity – ETS expressed as mJ/org/min. The available energy (Ea) was calculated by summing proteins, carbohydrates and lipids contents and is expressed as mJ/org.

For total protein and carbohydrate content measurements, the homogenate was mixed with trichloroacetic acid 15% (TCA) and samples incubated at -20ºC for 10 min. Following this step, samples were centrifuged (1000 *g*, 10 min, 4ºC) and the supernatant was separated to be used as the carbohydrate fraction. The pellet was resuspended in 625 µL sodium hydroxide (NaOH), incubated at 60ºC for 30 min, after which it was neutralized with 375 µL hydrochloric acid (HCl), and then used for measuring the protein fraction. Total protein was determined using the Bradford's reagent and the absorbance was measured at 590 nm in a microplate reader, using bovine serum albumin as a standard. Total carbohydrate content was determined by adding 150 µL of 5% phenol and 600 µL sulphuric acid (H_2SO_4) to 150 µL of sample. After 30 min incubation at 20ºC, the absorbance was measured at 492 nm using glucose as standard. The protein and carbohydrate content were expressed as mJ/org.

Total lipids were determined following the adapted protocol of Ferreira *et al.* (2015) previously described by De Coen and Janssen (1997). Here 500 µL of chloroform were added to the 300 µL of fraction and vortexed, after which 500 µL of methanol and 250 µL ultra-pure water were added and the samples centrifuged (1000 *g*, 5 min, 4ºC). The top phase was discarded and the bottom phase containing the lipid extraction was used for lipid measurements. These were diluted in 500 µL of H_2SO_4 and heated for 15 min at 200ºC. After cooling down, 1.5 mL ultra-pure water was added. The absorbance was measured at 375 nm. Lipid content is expressed as mJ/org.

Carbohydrates, proteins and lipids obtained were transformed into energetic equivalents using the energy of combustion described by Gnaiger (1983): 17,500 mJ/mg carbohydrate, 24,000 mJ/mg protein and 39,500 mJ/mg lipid.

For the electron transport activity, 150 µL of a buffer of 0.3 M Tris-HCl pH 8.5, 45% (w/v) Poly Vynil Pyrrolidone 459 µM MgSO4 and 0.6% (w/v) Triton X-100 were added to 300 µL homogenate. Samples were centrifuged (1000 *g*, 5 min, 4ºC) and supernatant was used as sample. In a microplate, to each 50 µL of the sample, 150 µL buffered substrate solution (0.13 M Tris HCl, 0.3% (w/v) Triton X-100, pH 8.5, 1.7 mM NADH and 250 µM NADPH) was added. The reaction started by adding 100 µL INT (p-IodoNitroTetrazolium, 8 mM). The absorbance was measured at 490 nm for 3 min. The amount of formazan formed was calculated using a molar extinction coefficient of 15,900 M^{-1} cm^{-1}.

2.4 Statistical Analysis

The mean value of the intrinsic rate of natural increase (*r*) was determined using the Jackknife method (Pestana *et al.*, 2010; Taberner *et al.*, 1993). The 50% lethal time (LT_{50}) values were calculated using a nonlinear regression with a three-parameter logistic function using SigmaPlot v11.0 software (Systat Software Inc., 2008). To compare the LT_{50} values obtained for Dph_Clean and Dph_CBZ, a generalized likelihood ratio test was applied using statistical package SPSS (SPSS 20.0.0, 2011). Normality was assessed using the Shapiro-Wilk test and homoscedascity using Levene's equal variance test (Systat Software Inc., 2008). GST and Ec data were square-root transformed to correct for normality. Significant differences between exposure/populations (Dph_Clean and Dph_CBZ) and generations (time) were checked for all endpoints (except longevity) using a two-way ANOVA with Bonferroni post-test; and generations (time) and exposure/population were used as fixed factors. The Two-way ANOVA were performed in SigmaPlot v11.0 software as well (Systat Software Inc., 2008). The R-squared (R^2) was calculated by dividing the sum of squares of each factor and of their interaction by the total sums of squares of the two-way ANOVAs (Hullett and Levine, 2003), to evaluate the percentage of variance accounted for each factor in the ANOVAs.

3. Results and Discussion

3.1 Chemical analysis

The results of the chemical analysis showed that CBZ concentration in the ASTM decreased over time, with a decay rate (*K0*) of 0.03/hour (SE=0.005), showing that only 18% of the initial concentration (7.2 µg/L) left after 48h (as also described in Chapter 2 - Silva *et al.* 2015).

3.2 Multigenerational effects

The physiological conditions of *D. magna* were measured by looking at their sensitivity towards exposure to the reference chemical potassium dichromate. The 24h-EC_{50} values obtained in daphnids from F0, F3, F6 and F12 were always within the recommended range of 0.6 mg/l to 2.1 mg/l (Table 5.1) (EN ISO 6341, 1996).

Table 5.1. Potassium dichromate 24h immobilisation tests in the F0, F3, F6 and F12 daphnids from clean medium (Dph_Clean) and exposed to carbendazim (Dph_CBZ) throughout generations. Data are expressed as 24h-EC_{50} values and corresponding standard error values between brackets.

Generation	**Dph_Clean 24h-EC_{50} (mg/L)**	**Dph_CBZ 24h-EC_{50} (mg/L)**
F0	1.26 (12.3)	-
F3	1.39 (0.11)	1.14 (8.69)
F6	0.83 (0.07)	0.90 (39.2)
F12	1.24 (6.88)	0.98 (0.05)

3.2.1 Reproduction and growth

Body length measured in *D. magna* (21 days old) was similar between both exposure/populations Dph_Clean and Dph_CBZ throughout the generations (F2, F4, F10 and F12) (two-way ANOVA, $F_{3,79} = 0.027$, $p>0.05$) (Fig. 5.2a and Table 5.2). In addition, no interaction between both factors was found (two-way ANOVA, $F_{3,79} = 1.15$, $p>0.05$). In a multigenerational experiment with *D. magna* and copper, length was already shown as a non-sensitive parameter with no significant differences observed for the sixth generation of daphnids acclimated to copper (concentrations up to 100 µg/L) (Bossuyt and Janssen, 2004).

The number of neonates differed between generations (two-way ANOVA, $F_{7,47}$ = 10.58, $p<0.001$) and between the exposure/populations Dph_Clean and Dph_CBZ (two-way ANOVA, $F_{1,47}$ = 6.27, $p<0.05$) (Table 5.2). However, no interaction between both factors was found, indicating that populations responded similarly throughout the generations (two-way ANOVA, $F_{7,47}$ = 0.85, $p>0.05$). Dph_CBZ produced always a lower number of neonates except in the F12 generation where this number was higher comparing with those from Dph_Clean (Fig. 5.2b). A hypothesis raised for the overall reduction in the reproductive output in Dph_CBZ might be related to the energy allocated to other processes rather than reproduction, for instance to detoxifying mechanisms (Hopkin, 1990). Therefore, less energy may be available for reproduction.

The intrinsic rate of natural increase (*r*) is a sensitive endpoint that can provide information at the population level (Buhl *et al.*, 1993). This endpoint (*r*) usually is more sensitive than considering only the number of neonates, because it integrates the number of neonates, number of mothers, number of broods and time (days) to the brood release. This enables also to bridge the gap on extrapolations from individuals to populations. However, this higher sensitivity was not observed in this study, with no significant differences for *r* between Dph_Clean and Dph_CBZ (two-way ANOVA, $F_{1,47}$ = 0.04, $p>0.05$) and neither an interaction between exposure/populations and generations (two-way ANOVA, $F_{7,47}$ = 1.29, $p>0.05$) (Fig. 5.2c and Table 5.2). Zalizniak and Nugegoda (2006) tested the effects of chlorpyrifos on three successive generations of *Daphnia carinata* and reported that no clear effects were observed for the intrinsic rate of natural increase (*r*) as observed in the present study. This might be related with a compensation between survival, fecundity and maturation time (Zalizniak and Nugegoda, 2006).

Some variability on data from unexposed daphnids was also observed (Fig. 5.2), which might be related with exposure conditions such as food quality (algae) and/or small variations in room temperature. Although all procedures/conditions are intended to be constant a slight inherent variability is likely to occur. In the work of Clubbs and Brooks (2007), a slight variance in the mean number of neonates/intrinsic rate of population growth was also shown even in controls of F0 and F1. Howsoever, as previously described all endpoints were assessed and compared in neonates originating from Dph_Clean and Dph_CBZ throughout the generations, to control inherent differences in organisms sensitivity as well (Loureiro *et al.*, 2010).

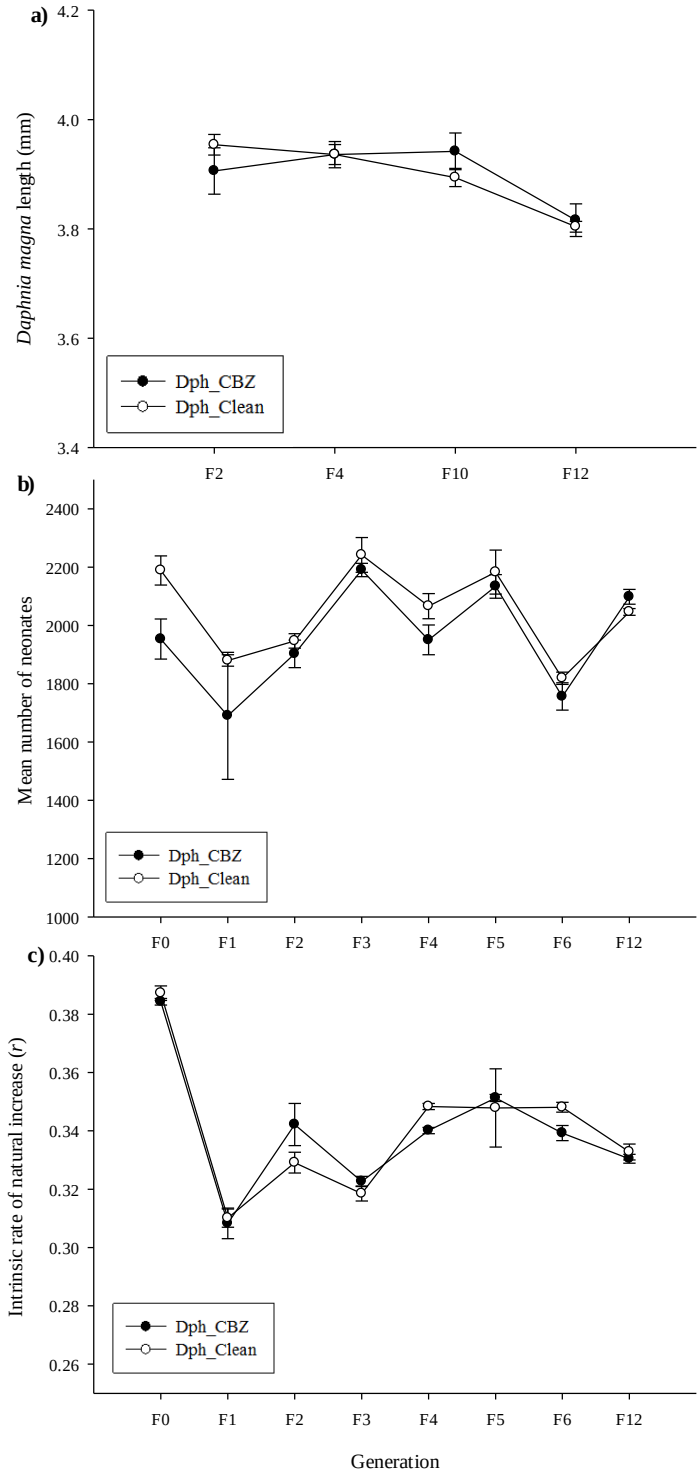

Figure 5.2. Life traits of *Daphnia magna* exposed throughout 12 generations to clean medium (Dph_Clean, white dots) and to carbendazim (Dph_CBZ, black dots): **a)** Body length (mm) **b)** mean number of neonates produced by 20 daphnids till the fifth brood **c)** intrinsic rate of natural increase (*r*). Data are expressed as mean values and standard error.

Table 5.2. Two-way ANOVA results for testing differences in two *Daphnia magna* populations regarding: factor 1- exposure/population (Dph_Clean *vs.* Dph_CBZ) and factor 2- generations (time), as well as the interaction of both factors on length, number of neonates and intrinsic rate of natural increase (*r*). In bold are the *p* values highlighted due to their statistical relevance (α=0.05).

	DF	Sum of squares	F	*p*-value	R^2
Length					
Generation	3	0.212	10.37	**<0.001**	0.29
Population	1	0.00018	0.027	0.871	0.00025
Generation x Population	3	0.0236	1.15	0.334	0.03
Reproduction (number of neonates)					
Generation	7	1074504.67	10.58	**<0.001**	0.63
Population	1	91002.08	6.27	**0.018**	0.053
Generation x Population	7	86789.25	0.85	0.552	0.051
Intrinsic rate of natural increase (*r*)					
Generation	7	0.0218	51.64	**<0.001**	0.90
Population	1	0.00000243	0.040	0.842	0.0001
Generation x Population	7	0.000542	1.29	0.289	0.022

3.2.2 Longevity

Longevity was assessed in the F0, F3, F6 and F12 generations, in both Dph_Clean and Dph_CBZ and the 50% lethal time (LT_{50}) in days was calculated. In the F0 generation, the pattern for longevity was similar between Dph_Clean and Dph_CBZ (Fig. 5.3a), which was reinforced by the similar LT_{50} values: 60.66 days (SE=0.39) and 60.71 days (SE=0.43), respectively (Table 5.3), with no significant differences in slopes of the probit regressions between both LT_{50} values ($X^2_{df=1}$ = 0.008, p>0.05). When analysing the F3 and F6, the LT_{50} values changed and were significantly different between both exposure/populations: for F3 ($X^2_{df=1}$= 56.39, p<0.05) and F6 ($X^2_{df=1}$= 409.92, p<0.05), being higher in Dph_CBZ comparing with Dph_Clean. After twelve generations (F12), the longevity appeared to be even more affected by CBZ, with a LT_{50} value of 57.86 days (SE=0.20), significantly lower than for Dph_Clean which was of 76.18 days (SE=0.20) ($X^2_{df=1}$= 593.93, p<0.05) (Table 5.3). This decrease in longevity of Dph_CBZ might be related with a cumulative effect of CBZ, suggesting that sufficiently long-term exposure for several generations to this compound affected the longevity of daphnids. Survival has been evaluated in daphnids in multigenerational tests, however this assessment is usually carried out only until the 21 day (corresponding to a standard reproduction test with *Daphnia*) (Chen *et al.*, 2013; Sánchez *et al.*, 2004; Tanaka and Nakanishi, 2002). Chen *et*

al. (2013) observed that the pesticide pentachlorophenol caused an earlier mortality in F2 comparing with F0 daphnids, representing an enhanced toxic effect in the F2 generation. Increase in sensitivity due to a continuous exposure probably results from chemical bioaccumulation or transgenerational reductions in fitness (Kimberly and Salice, 2014).

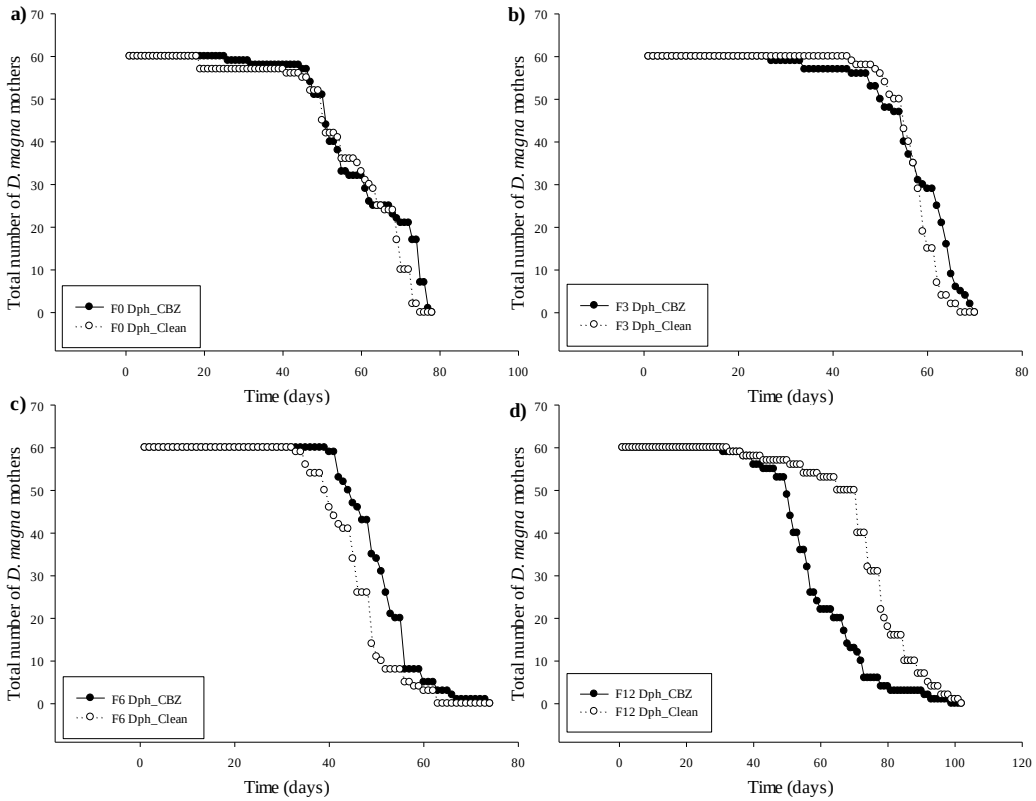

Figure 5.3. Longevity of *Daphnia magna* populations exposed to clean medium (Dph_Clean, white dots) and to carbendazim (Dph_CBZ, black dots) in several generations: **a)** F0 generation **b)** F3 generation **c)** F6 generation and **d)** F12 generation. Longevity is expressed as cumulative survival.

Table 5.3. LT_{50} values (in days) and corresponding standard error values between brackets, calculated for F0, F3, F6 and F12 in clean medium (Dph_Clean) and exposed to carbendazim (Dph_CBZ).

Generation	**Dph_Clean LT_{50}**	**Dph_CBZ LT_{50}**
F0	60.66 (0.39)	60.71 (0.43)
F3[a]	57.47 (0.07)	58.80 (0.20)
F6[a]	45.48 (0.13)	50.70 (0.11)
F12[a]	76.18 (0.20)	57.86 (0.20)

[a] Denotes significant differences between exposures (clean medium and daphnids in carbendazim) within the same generation and according to a generalized likelihood-ratio test (X^2_{df}> 3.84; p<0.05).

3.2.3 Comet assay

A major goal of the present study was to evaluate the underlying mechanisms leading to effects at higher organizational levels and also to understand whether or not such subcellular effects could have multigenerational effects. In a previous work, CBZ was found to induce DNA damage to *D. magna* (Chapter 2 - Silva *et al.*, 2015) so it can be hypothesized that such damages could be transmitted to the offspring in this parthenogenetically reproducing organism.

The percentage of DNA damage was evaluated in the F3 and F13 generations of Dph_Clean and Dph_CBZ. Both populations differed on their percentage of DNA damage (two-way ANOVA, $F_{1,15}$ = 76.647, $p<0.001$), with Dph_CBZ presenting a higher percentage than those from Dph_Clean in both generations (Fig. 5.4 and Table 5.4). The factor exposure/population explained the majority of the total variation, 71% (R^2 =0.71). Interaction of both factors, generation and population, was observed, indicating that populations responded differently throughout the generations (two-way ANOVA, $F_{1,15}$ = 10.778, $p<0.05$) (Table 5.4). In addition, Dph_Clean exhibited a similar percentage of DNA damage in both F3 and F13 generation ($p>0.05$, two-way ANOVA, Bonferroni post hoc test), whereas Dph_CBZ showed an increased percentage of DNA damage from the F3 to the F13 generations ($p<0.05$, two-way ANOVA, Bonferroni post hoc test) (Fig. 5.4). Similarly, Antiezar and Jha (2004) observed that *D. magna* exposed throughout generations to benzo(a)pyrene, presented genetic damage that was most probably transmitted to the offspring. The study conducted by Parisot *et al.* (2015) provided also evidences that *D. magna* exposed to low doses of gamma radiation show DNA alterations that were accumulated and transmitted throughout the three generations tested. However, it is important to note, that genotoxicity also depends on the efficiency of several repair mechanisms (Jha, 2008). Another hypothesis that could explain the increase in DNA damage observed in the present study over the thirteen generations (F13) is that the repair of DNA strand breaks were probably being less effective (reduced or slower repair) as a consequence of the exposure to the toxic compound (Collins *et al.*, 1995).

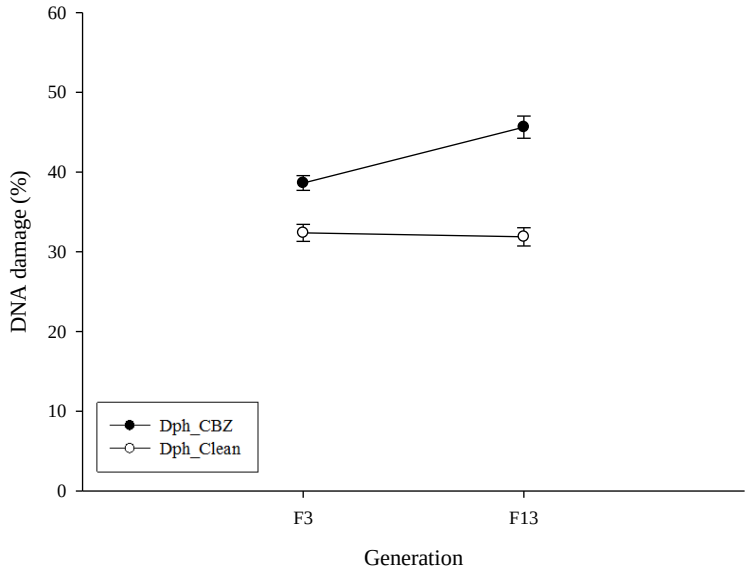

Figure 5.4. DNA damage (%) in *Daphnia magna* cells from F3 and F13 exposed to clean medium (Dph_Clean, white dots) and exposed to carbendazim (Dph_CBZ, black dots). Data are expressed as mean values and standard error.

Table 5.4. Two-way ANOVA results for testing differences in two *Daphnia magna* populations regarding: factor 1- exposure/population (Dph_Clean *vs.* Dph_CBZ) and factor 2- generations (time), as well as the interaction of both factors on DNA damage (%). In bold are the *p* values highlighted due to their statistical relevance (α=0.05).

	DF	**Sum of squares**	**F**	***p*-value**	**R^2**
DNA damage (%)					
Generation	1	42.250	8.096	**0.015**	0.075
Population	1	400.00	76.647	**<0.001**	0.71
Generation x Population	1	56.250	10.778	**0.007**	0.10

3.2.4 Biochemical assays

Several endpoints have been evaluated in multigenerational tests with *Daphnia* sp. (Jacobasch *et al.*, 2014; Kim *et al.*, 2012; Sánchez *et al.*, 2004), however the study of effects in physiological pathways, such as neurotransmission capabilities, detoxification potential or antioxidant capacity is less common.

Carbamate pesticides are known to inhibit the ChE activity in *D. magna* (Barata et al., 2004). Though, in the present study ChE levels were significantly higher for the population maintained in CBZ (Dph_CBZ) than for those in clean medium (Dph_Clean)

(two-way ANOVA, $F_{1,39} = 11.737$, $p<0.05$) (Table 5.5), at all generations (Fig. 5.5a). An increase in the ChE activity in *D. magna* exposed to low concentrations of cadmium and the carbamate propoxur was already reported (Jemec *et al.*, 2007a; Printes and Callaghan, 2004). Increase of ChE at low doses might be explained by compensatory mechanisms after the disruption of homeostasis (Calabrese and Baldwin, 2003). Andrade *et al.* (2016) observed an increase in ChE activity after exposure of zebrafish (*Danio rerio*) embryos to CBZ, which the authors hypothesized to be related with apoptosis mechanisms. Although the exact mechanisms are not yet understood, indirect evidences that ChE participates in the regulation of apoptosis and cell proliferation has been theorised (Jiang and Zhang, 2008). In turn, CBZ exposure has been connected to the induction of gene expression related with apoptosis (Jiang *et al.*, 2014). In the present thesis, genomics' tool were applied upon a multigenerational exposure and it was demonstrated that *D. magna* exposed to CBZ (F0 and F12 generation) showed deregulation in several genes involved in apoptosis as well (Chapter 6). A similar mechanism related with mediation of cell apoptosis could be playing a role in the ChE activity increase observed in the present study, however additional studies should be performed to confirm this hypothesis. Both factors, generation and exposure/population, interacted, indicating that populations responded differently throughout the generations (two-way ANOVA, $F_{3,39} = 3.393$, $p<0.05$) (Table 5.5). However throughout the successive generations (F0 to F12), there was an overall trend for the attenuation of this effect (Fig. 5.5a). From F0 to F12, Dph_CBZ population presented a decrease in ChE activity (Fig. 5.5a). In the present study, variation in ChE activity was observed throughout generations, including in Dph_Clean. Variation in ChE activity amongst individuals of the same species has been observed for *D. magna* in previous studies, including under control conditions. In the literature and for *D. magna*, ChE levels varied from 0.034 to approximately 1.5 nmol/mg prot/min (Jemec *et al.*, 2007a; Qi *et al.*, 2013), which is in accordance with the ChE activity reported in the present study for daphnids kept in clean medium.

Although the main toxicity mechanism of carbamates is usually through ChE inhibition, exposure to carbamate pesticides has also been shown to trigger other toxicity effects such as oxidative stress, by inducing generation of reactive oxygen species (ROS) (Milatovic *et al.*, 2006). CAT is an antioxidant enzyme, which is responsible for breaking down hydrogen peroxide into water and molecular oxygen (Claiborne, 1985). CAT activity

values for *D. magna* control groups from previous studies ranged from 62.4 µmol/mg prot/min (for daphnids with 22 days) to 250 µmol/mg prot/min (for daphnids with 6 days) (Barata *et al.*, 2005; Jemec *et al.*, 2007b). In the present work, CAT levels determined for Dph_Clean were slightly lower comparing with the values reported in literature, however several factors might cause variability, including for instance the type of food to feed the daphnids (*e.g.* algae species), daphnids age and experimental conditions (*e.g.* temperature or photoperiod). Comparing both populations, Dph_Clean and Dph_CBZ did not differed statistically for CAT levels (two-way ANOVA, $F_{1,37} = 0.348$, $p>0.05$), though both factors interacted, meaning that populations responded differently throughout the generations (two-way ANOVA, $F_{3,37} = 10.271$, $p<0.001$) (Table 5.5). Two patterns were observed when comparing populations in the same generation: in the F0 and F6 generation an increase in the activity of this enzyme was observed; and in the F3 and F12 generation a decrease for Dph_CBZ comparing with Dph_Clean. The initial response of Dph_CBZ (F0 generation) consisted on an increase in this antioxidant enzyme when comparing with Dph_Clean population, which might indicate a response mechanism to oxidative stress (Vega and Pizarro, 2000). Following this increase in F0, a decrease in CAT activity was generally observed, which had already been described for other species, including the earthworm *Lumbricus rubellus* exposed to pyrene (Brown *et al.*, 2004). A decrease in CAT activity was observed as well for the herb fenugreek *Trigonella foenum-graecum* exposed to CBZ (Sangeetha, 2010), which was justified by a possible increase on the use of these antioxidant to combat the ROS highly produced during the oxidative stress. Reduction in CAT activity was also observed in fish tissues (Palanikumar *et al.*, 2014), rats (Adedara *et al.*, 2013) and goats (Prashantkumar *et al.*, 2013) after exposure to CBZ. Other possibility for the CAT activity decrease (in Dph_CBZ comparing with Dph_Clean) is the simultaneous activation of another antioxidant defense mechanism, *e.g.* reduced glutathione (GSH), which is also involved in the removal of hydroperoxides (*e.g.* H_2O_2) such as CAT (Sies, 1993; Wu *et al.*, 2011). This mechanism of GSH oxidative protection occurs by its oxidation (mediated by H_2O_2) into glutathione dissulfide (GSSG), therefore reducing the amount of substrate available to induce CAT, which may be translated into lowered CAT activity (Wu *et al.*, 2011). Nevertheless, to better understand what is happening, additional studies should be performed. Considering the Dph_Clean population, the CAT activity was similar comparing the F0 and F12 generation (Fig. 5.5b).

On the other hand, in Dph_CBZ population, from generation F0 to F12, it was noticed an overall decrease on the CAT activity, with the only exception for the F3 generation (CAT activity in F3 decreased comparing with F0, yet in F6 CAT activity increased again) (Fig. 5.5b).

Levels of the detoxification enzyme GST were different between both populations (Dph_Clean and Dph_CBZ) (two-way ANOVA, $F_{1,33} = 4.557$, $p<0.05$) (Fig. 5.5c and Table 5.5). Both factors, generation and exposure/population, interacted, indicating that populations responded differently throughout the generations (two-way ANOVA, $F_{3,33} = 2.951$, $p<0.05$) (Table 5.5). This enzyme plays an important role in cellular detoxification processes of several chemicals and defense against peroxidative products of DNA (Henson *et al.*, 2001). Pesticides can promote the consumption of glutathione by the organisms through a GST-catalyzed reaction in detoxification, and therefore they can induce the activity of GST to protect the organism (Ezemonye and Tongo, 2010; Timur *et al.*, 2002). A multigenerational experiment with *D. magna* exposed to microcystins showed that the offspring of a parental generation pre-exposed for 7 days to microcystins had a higher GST activity than control daphnids (Ortiz-Rodriguez *et al.*, 2012). The reported GST values in the literature for control groups in *D. magna* are in accordance with the ones from the present study (from 42 nmol/mg prot/min (*D. magna* with 7 days), to 70 nmol/mg prot/min (*D. magna* with 21 days) and reaching 235.2 nmol/mg prot/min) (Borgeraas and Hessen, 2002; Chen *et al.*, 2005; Domingues *et al.*, 2015).

LPO differed between both exposure/populations (Dph_Clean and Dph_CBZ) (two-way ANOVA, $F_{1,39} = 12.957$, $p<0.001$) and there was an interaction between generations and exposure/population (two-way ANOVA, $F_{3,39} = 6.612$, $p<0.001$) (Fig. 5.5d and Table 5.5). In the F0 generation, a decrease in LPO in Dph_CBZ was observed, comparing with Dph_Clean. Since this decrease was concomitant with an increase in the activity of ROS-scaveging enzymes (mainly CAT), it is possible that it resulted from a prompt antioxidant response by the organisms of this generation. Vernouillet *et al.* (2010) observed a similar decrease in lipid peroxidation when exposed the crustacean *Thamnocephalus platyurus* to the pharmaceutical carbamazepine and suggested that carbamazepine might have preventing fatty acid oxidation in the membranes, by acting as a radical scavenger or by directly downregulate the cytosolic phospolipase A_2 activity. However, in the F6 generation differences in LPO between Dph_Clean and Dph_CBZ

were atenuated (Fig. 5.5d). The crab *Chasmagnathus granulate* exposed to UV-A and UV-B radiation showed no significant differences for LPO content (Gouveia *et al.*, 2005). However, in the last generation tested (F12), there was a slightly increase in LPO for Dph_CBZ comparing with Dph_Clean. This seems to indicate an imbalance in organisms redox equilibrium towards a situation of oxidative stress as was previously described in several organisms (including the european eel and collembola) when exposed to harbor water, carbamazepine, fluoxetine and nanoparticle fullerene C60 (Ahmad *et al.*, 2004; Oliveira *et al.*, 2015; Zhu *et al.*, 2006). Literature for *D. magna* regarding LPO data by measuring TBARS is scarce.

Palanikumar *et al.* (2014) evaluate the DNA damage (using the micronuclei assay) in fish tissues after exposure to CBZ and chlorpyrifos, and they concluded that a relationship between DNA damage and the fluctuation in antioxidant enzymes responses might exist. Herein, an increase in DNA damage was observed from generation F3 to F13, however such straight relationship with antioxidant enzymes could not be established.

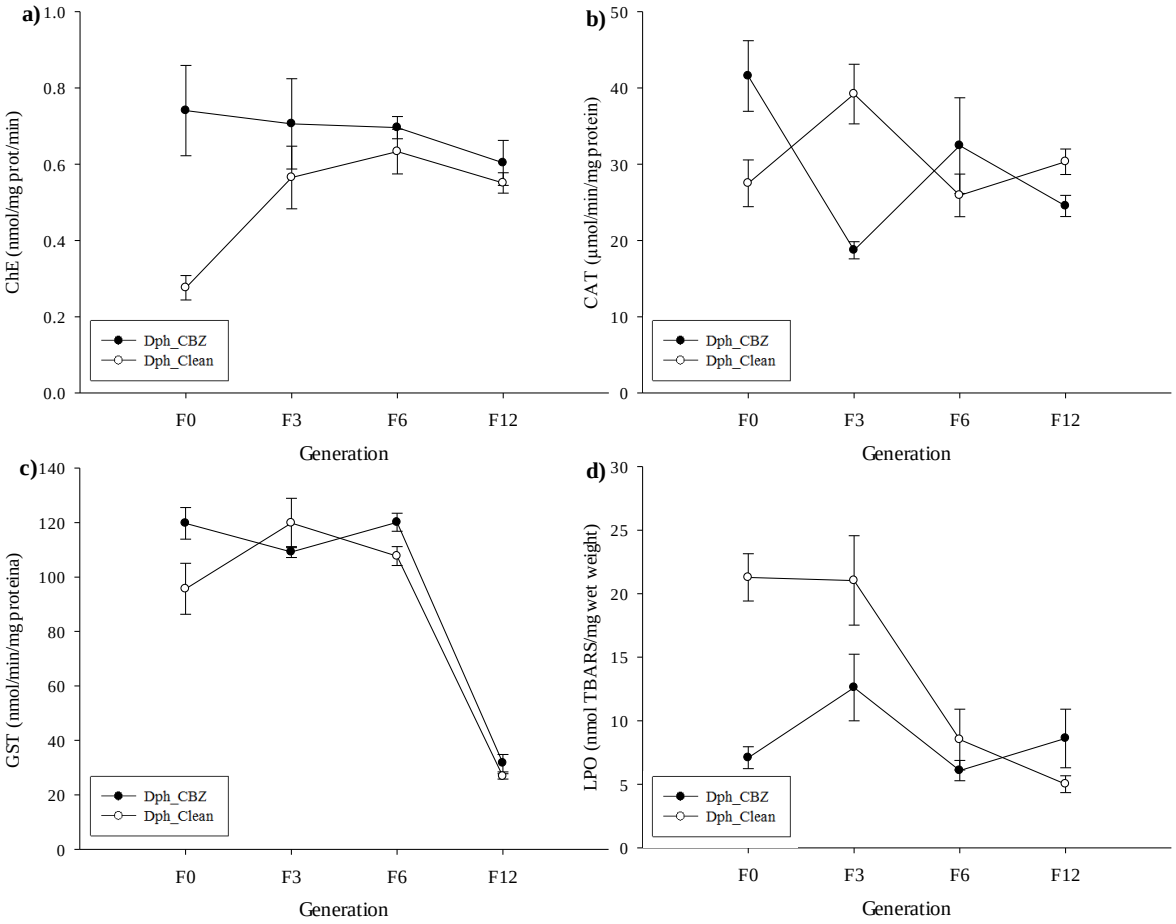

Figure 5.5. Biomarkers activities in *Daphnia magna* exposed to clean medium (Dph_Clean, white dots) and to carbendazim (Dph_CBZ, black dots) throughout generations: **a)** Cholinesterase (ChE) activity **b)** Catalase (CAT) activity **c)** Glutathione *S*-transferase (GST) activity and **d)** Lipid peroxidation (LPO). Data are expressed as mean values and standard error.

Table 5.5. Two-way ANOVA results for testing differences in two *Daphnia magna* populations regarding: factor 1- exposure/population (Dph_Clean *vs.* Dph_CBZ) and factor 2- generations (time), as well as the interaction of both factors on Cholinesterase (ChE) activity, Catalase (CAT), Glutathione *S*-transferase (GST) activity and Lipid peroxidation (LPO) rate. In bold are the *p* values highlighted due to their statistical relevance (α=0.05).

	DF	Sum of squares	F	*p*-value	R^2
Cholinesterase					
Generation	3	0.143	1.724	0.182	0.088
Population	1	0.325	11.737	**0.002**	0.20
Generation x Population	3	0.281	3.393	**0.030**	0.17
Catalase					
Generation	3	280.497	1.704	0.187	0.077
Population	1	19.083	0.348	0.560	0.005
Generation x Population	3	1690.434	10.271	**<0.001**	0.46
Glutathione *S*-transferase					
Generation	3	184.320	152.923	**<0.001**	0.91
Population	1	1.831	4.557	**0.041**	0.009
Generation x Population	3	3.557	2.951	**0.048**	0.018
Lipid peroxidation					
Generation	3	750.978	11.261	**<0.001**	0.34
Population	1	288.014	12.957	**0.001**	0.13
Generation x Population	3	440.941	6.612	**0.001**	0.20

3.2.5 Energy reserves experiments

When under stress and in order to survive, organisms undergo numerous alterations at a low level of biological organization. These alterations include for instance metabolic changes that may end up affecting their energy-reserve fraction and energy consumption (Jeon *et al.*, 2013; Vandenbrouck *et al.*, 2009).

Considering the multigenerational effects observed in several life traits, some energy-related parameters were measured in different generations as an attempt to detect and track possible CBZ induced changes in resource allocation.

Carbohydrates, which are considered the first energy fraction to be consumed, presented an almost similar pattern between populations Dph_Clean and Dph_CBZ (two-way ANOVA, $F_{1,23}$ = 0.167, p>0.05) (Fig. 5.6a and Table 5.6). After three generations (F3), carbohydrates in Dph_CBZ were slightly lower comparing with Dph_Clean. Depletion of carbohydrates was observed in a different species, *Enchytraeus albidus*, throughout different treatments of CBZ (Novais and Amorim, 2013). However, no interaction between both factors, generations and exposure/population, occurred for carbohydrates (two-way ANOVA, $F_{3,23}$ = 2.202, p>0.05) (Table 5.6). Carbohydrates values

obtained in the present study for Dph_Clean are within the ones found in literature. For *D. magna,* carbohydrates reported values in controls varied between 199 to 2054 mJ/organism (for neonates <24h exposed for 48h/96h) (De Coen and Janssen, 1997).

With respect to the protein content, a similar pattern was also found between populations Dph_Clean and Dph_CBZ (two-way ANOVA, $F_{1,23} = 0.00115$, $p>0.05$) (Fig. 5.6c and Table 5.6). In addition, no interaction between both factors, generations and exposure/population, occurred (two-way ANOVA, $F_{3,23} = 3.129$, $p>0.05$) (Table 5.6). The Dph_Clean population presented protein values ranging from 2000 mJ/org to 3000 mJ/org. In the F6, protein values for Dph_CBZ were higher comparing with those from Dph_Clean. Several authors reported an increase in protein content for several species, namely for *D. magna*, *Danio rerio* and *E. albidus* respectively exposed to lindane, effluents and also CBZ (De Coen and Janssen, 2003a; Novais and Amorim, 2013; Smolders *et al.*, 2003). A hypothesis for this increase was raised by several authors and is related to the induction of protein synthesis used for detoxification mechanisms (Novais and Amorim, 2013; Smolders *et al.*, 2003). However, in the F12 generation protein values become similar between Dph_Clean and Dph_CBZ (Fig. 5.6c). In literature, protein values for *D. magna* in control situation ranged from to 1694 mJ/organism to 5518 mJ/organism (exposed for 48h) (De Coen and Janssen, 1997). Once again, values obtained in this work for Dph_Clean are in line with the ones found in literature.

For the lipids reserves, both populations Dph_Clean and Dph_CBZ did not differ in their contents (two-way ANOVA, $F_{1,22} = 0.113$, $p>0.05$) and no interaction between both factors (generations and exposure/population) was found (two-way ANOVA, $F_{3,22} = 1.334$, $p>0.05$) (Fig. 5.6b and Table 5.6). However, when considering Dph_CBZ, a slight continuous decrease can be noticed throughout the generations (Fig. 5.6b). A decrease in lipids reserves might be possibly related with an increase on energy demand compensating for the CBZ stress in this long-term exposure. Jeon *et al.* (2013) observed a decrease in the lipid content when *D. magna* was exposed to the carbamate pesticide, carbaryl. Additionally, in crustaceans, lipids serve as membrane building materials and energy storage molecules and specifically in cladocerans, lipids are also known to be involved in egg production (Goulden and Henry, 1987). In fact, in the present study the intrinsic rate of natural increase (*r*) and the number of neonates produced in the F6 generation was lower than in the F0, F3 and F12, in contrast with the energy parameters, thus showing a

disinvestment in reproduction. The lipid levels obtained in this work for daphnids in clean medium (Dph_Clean) are within the same range reported in literature for the *Daphnia* species (approx. 1000 mJ/organism) (Bergman Filho *et al.*, 2011).

Kim *et al.* (2014) studied the effects of tetracycline in four generations of *D. magna*; depletions in proteins, carbohydrates and lipid reserves were found in consequence of the stress caused by tetracycline. However, throughout the generations, these reductions were recovered (comparing with the control group), suggesting some adaptation (Kim *et al.*, 2014). Muyssen and Janssen (2004) observed that *D. magna* showed an increase in tolerance after two generations of exposure to cadmium, however, this increase disappeared with an extended acclimation time.

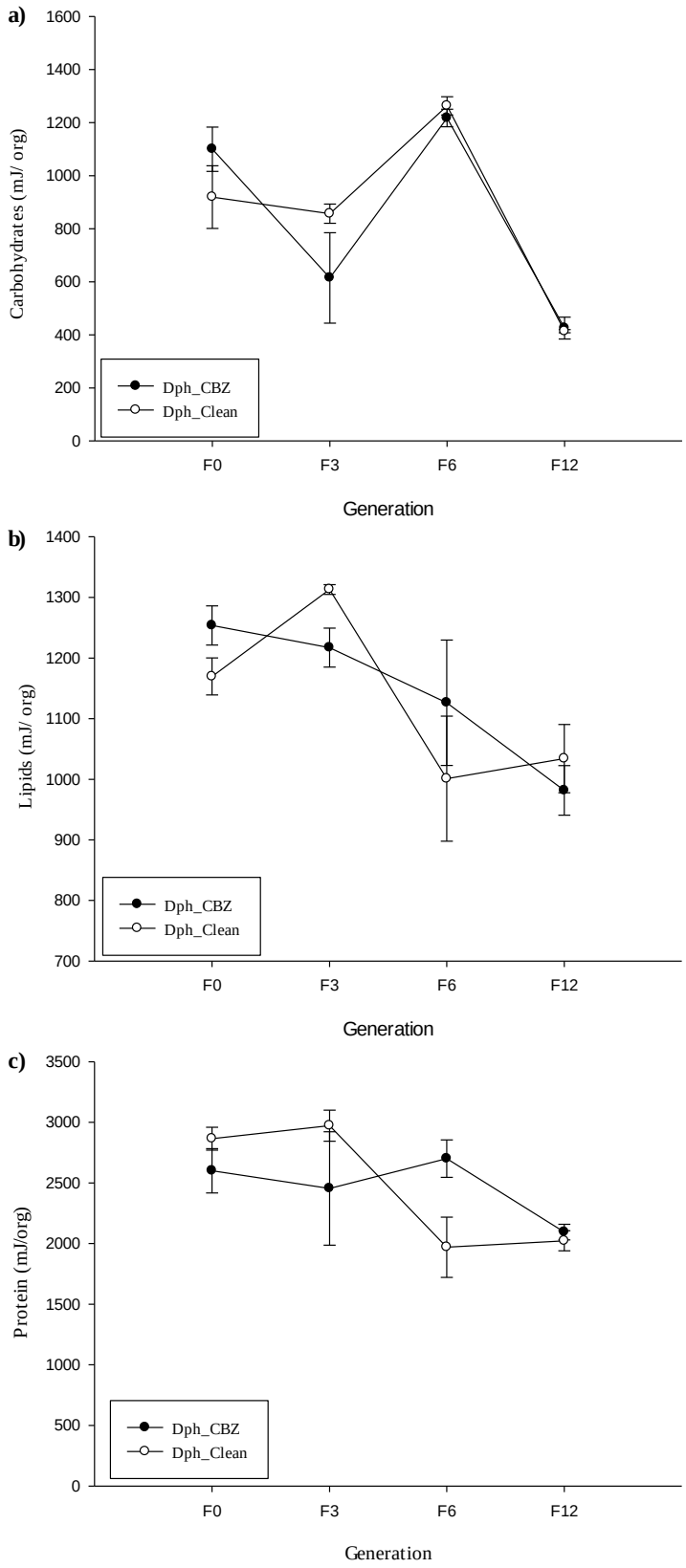

Figure 5.6. Energy-related parameters in *Daphnia magna* exposed to clean medium (Dph_Clean, white dots) and to carbendazim (Dph_CBZ, black dots) throughout generations: **a)** Carbohydrates **b)** Lipids and **c)** Protein contents. Data are expressed as mean values and standard error.

Table 5.6. Two-way ANOVA results for testing differences in two *Daphnia magna* populations regarding: factor 1- exposure/population (Dph_Clean *vs.* Dph_CBZ) and factor 2- generations (time), as well as the interaction of both factors on carbohydrates, lipids and proteins. In bold are the *p* values highlighted due to their statistical relevance (α=0.05).

	DF	Sum of squares	F	*p*-value	R^2
Carbohydrates					
Generation	3	2256777.654	36.360	**<0.001**	0.83
Population	1	3462.435	0.167	0.688	0.0013
Generation x Population	3	136646.115	2.202	0.128	0.05
Lipids					
Generation	3	243488.430	6.955	**0.004**	0.53
Population	1	1316.295	0.113	0.742	0.0029
Generation x Population	3	46718.387	1.334	0.300	0.10
Proteins					
Generation	3	1892078.747	4.502	**0.018**	0.35
Population	1	161.152	0.00115	0.973	2.95×10^{-5}
Generation x Population	3	1315322.675	3.129	0.055	0.24

No differences were observed between lipid, protein and carbohydrates contents in Dph_Clean and Dph_CBZ (Table 5.6), therefore no differences were expected for the Energy available (Ea). Indeed, both populations did not significantly differ (two-way ANOVA, $F_{1,23} = 0.0961$, $p>0.05$) (Table 5.7) and no interaction between generations and populations occurred (two-way ANOVA, $F_{3,23} = 1.394$, $p>0.05$) (Table 5.7). The present results showed that for the concentration used in the present study, no significant effect on the Ea as well for Energy consumption (Ec) were observed (two-way ANOVA, $F_{1,23} = 1.759$, $p>0.05$) (Fig. 5.7 and Table 5.7). However, generation and exposure/population factors interacted for Ec (two-way ANOVA, $F_{3,23} = 3.305$, $p<0.05$) (Table 5.7). The fact that no differences between population Dph_Clean and Dph_CBZ were observed suggests that rather than altering patterns of Ec, the multigenerational effects reported for previous endpoints must have been due to differences in allocation. While length seemed to be unaffected throughout the multigenerational experiment, a trade-off seemed to have occurred for instance in organisms longevity. Considering the close relationship between size and reproductive potential and the small differences registered in the number of juveniles produced within each generation, this seems to indicate that exposed daphnids did not favoured the reproductive output. Several examples can be found in literature where *D. magna* showed similar ability to switch its life history responses to stressors (Minguez *et al.*, 2015). At the population level, results showed no great effect on the

intrinsic rate of natural increase (*r*) by CBZ contaminated mothers. However population-level endpoints should not be disregarded in ecotoxicity studies and further on risk assessments, thus making these procedures more ecologically relevant (Forbes and Calow, 1999).

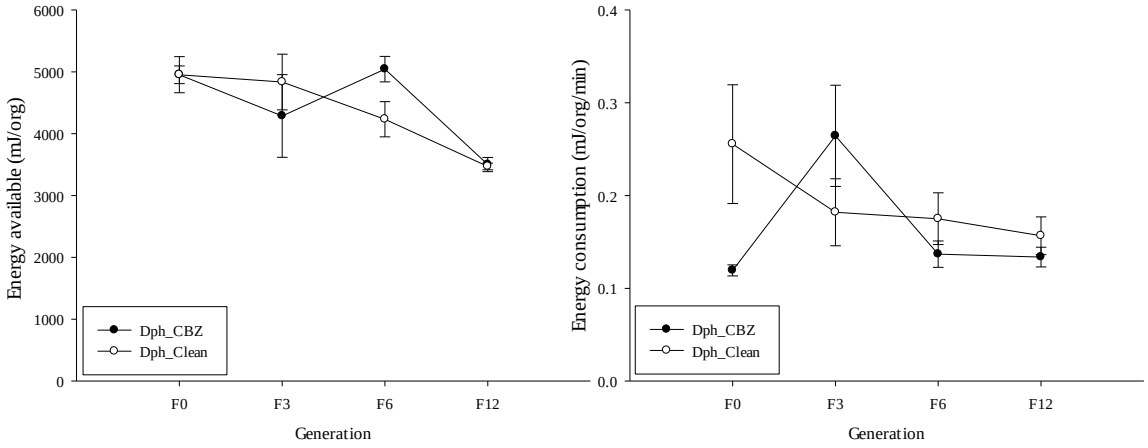

Figure 5.7. Energy-related parameters on individuals of *Daphnia magna* in clean medium (Dph_Clean, white dots) and exposed to carbendazim (Dph_CBZ, black dots) throughout generations. Data are expressed as mean values and standard error.

Table 5.7. Two-way ANOVA results for testing differences in two *Daphnia magna* populations regarding: factor 1- exposure/population (Dph_Clean *vs.* Dph_CBZ) and factor 2- generations (time), as well as the interaction of both factors on energy available and energy consumption after a long-term exposure to carbendazim. In bold are the *p* values highlighted due to their statistical relevance (α=0.05).

	DF	Sum of squares	F	*p*-value	R^2
Energy available					
Generation	3	7347657.730	7.296	**0.003**	0.52
Population	1	32271.840	0.0961	0.761	0.0022
Generation x Population	3	1404016.980	1.394	0.281	0.099
Energy consumption					
Generation	3	0.0261	2.030	0.150	0.18
Population	1	0.00753	1.759	0.203	0.052
Generation x Population	3	0.0425	3.305	**0.047**	0.29

Some allocation of energy reserves to detoxification processes seemed to have occurred, as GST activity increased along with a significant higher LT_{50} value and jointly with a lowest number of neonates for instance for the F6 Dph_CBZ. Although in literature effects of CBZ in the energy related parameters show a decrease in their content (Sancho *et*

al., 2009), it should be noted that the low concentration used in this study showed no significant changes in the intrinsic rate of natural increase (*r*) or growth (length) which is in accordance with energy reserves results.

In the same multigenerational study of Kim *et al.* (2014), an increase in energy consumption throughout the generations of *D. magna* was found, possibly as consequence of augmented energy demands needed for defense mechanisms (Kim *et al.*, 2014).

4. Conclusions

The present study demonstrated that a continuous exposure to CBZ did not cause changes in the intrinsic rate of natural increase (*r*) and length of the cladoceran *D. magna*. However, a long lasting exposure to CBZ affected their longevity, with a notorious decrease in the lifespan in daphnids exposed for 12 generations to CBZ. Additionally and considering subcellular effects, an increase in DNA damage was observed in daphnids exposed to CBZ (from F3 to F13 generation). The biomarkers ChE, GST and LPO showed differences between clean and exposed populations, however no clear pattern of increase or decrease in tolerance was observed. Energy related-parameters evaluated in this long-term exposure to CBZ showed no significant differences between both populations.

The current work point out to the importance that should be given to multigenerational experiments, as this scenario of a long-term exposure is highly likely to occur. Despite the fact that organisms exposed to CBZ did not show any difference in terms of intrinsic rate of natural increase (*r*)/growth, they were affected in terms of their DNA and longevity, which is an extremely important factor that could have consequences to the populations.

Acknowledgments

This work was supported by the project RePulse- Responses of *Daphnia magna* exposed to chemical pulses and mixtures throughout generations (FCOMP-01-0124-FEDER-019321; Refª. FCT PTDC/AAC-AMB/117178/2010), by funding FEDER through COMPETE- Programa Operacional Factores de Competitividade, and by the Portuguese Science Foundation (FCT) through CESAM: UID/AMB/50017/2013. Ana Rita Rego Gouveia Silva was funded by the project "MARPRO- Conservation of marine protected species in Mainland Portugal" through the doctoral fellowship (BD/UI88/5534/2011), Diogo Cardoso was funded by the doctoral grant (PD/BD/52569/2014) and Andreia Cruz was funded by an individual post-doctoral grant (BPD/UI88/2886/2013), within the project "Sustainable Use of Marine Resources" - MARES (CENTRO-07-ST24-FEDER-002033), financed by QREN, Mais Centro- Programa Operacional Regional do Centro e União Europeia/ Fundo Europeu de Desenvolvimento Regional.

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Supplementary data

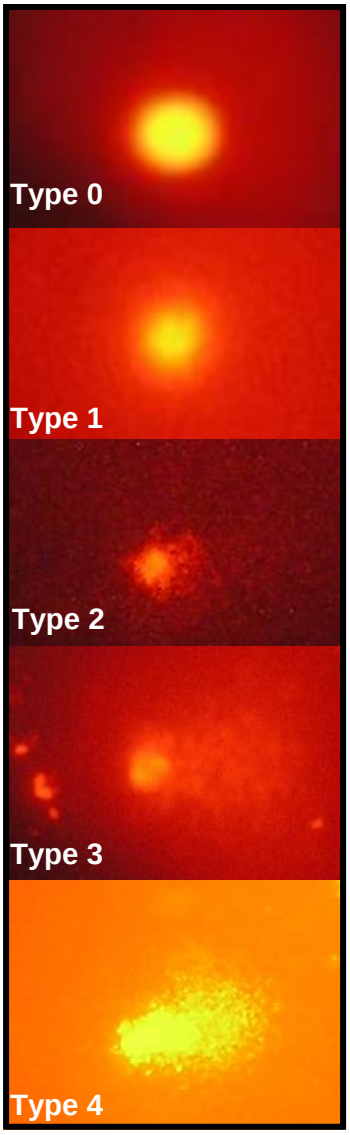
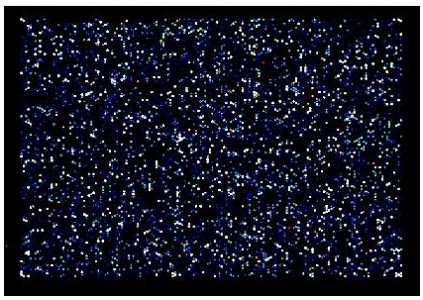


Figure 5.1 SD. Comet type scale used in daphnid cells.

Chapter 6

Gene transcription analysis of Daphnia magna exposed to carbendazim: a multigenerational approach



Gene transcription analysis of *Daphnia magna* exposed to carbendazim: a multigenerational approach

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Abstract

Pesticides are continuously released into the environment, with possible long-term consequences on aquatic organisms. One of the pesticides still applied in several crops in some countries is the fungicide carbendazim, ending up in surface waters with concentrations reaching 5 µg/L. *Daphnia magna* (clone k6) was used in this study as a model organism and it was exposed to a sublethal concentration of carbendazim (5 µg/L) for twelve generations. Gene expression alterations induced by this compound were assessed in the F0 and F12 generations using a *D. magna* custom microarray. Results revealed that carbendazim caused changes at the gene expression level in both generations. Genes involved in response to stress, DNA replication/repair, neurotransmission, protein biosynthesis, ATP production, lipids and carbohydrates metabolism were the most affected in both F0 and F12, although a lower number of differentially expressed genes was observed in the F12 generation exposed to carbendazim. The exposure of daphnids to carbendazim did not cause a stable change in gene expression from F0 to F12 generations. Effects at the gene expression level were early detected at the F0 generation after a short-time exposure (10 days), highlighting the advantages of using high throughput tools as early warning analysis, which can add value in risk assessment procedures.

Key words: *Daphnia magna*, carbendazim, multigenerations, microarrays

1. Introduction

Carbendazim (CBZ, methyl-2-benzimidazole carbamate) is used as an active ingredient in fungicides to eradicate several pathogens. It is applied in agriculture for protecting crops, vegetables, fruits, ornamental plants and as a preservative in textiles or paints, among other functions (Campos *et al.*, 2015; Davidse, 1987; Selmanoğlu *et al.*, 2001). Despite being prohibited in some countries, carbendazim is still autorized at a national level in some European countries including Portugal and the United Kingdom (EU Pesticide Database, 2015). It has been detected in surface waters of Thailand, Spain (Guadalquivir river basin) and Chile (Traiguén river basin) at concentrations of nearly 5 µg/L, contributing, therefore, to the contamination of the aquatic environment and possibly posing risk to non-target organisms (Chatupote and Panapitukkul, 2005; Masia *et al.*, 2013; Palma *et al.*, 2004). The environmental release of carbendazim might be continuous and not restricted to one season, since it is applied in several crops and in different seasons (EU Pesticide Database, 2015).

The cladoceran *Daphnia magna* is a recommended test species and one of the most studied organisms in ecotoxicology (OECD, 2008). Daphnids have an important role in ecological food webs and reproduce by parthenogenesis, making this species a suitable organism for experimental genetic studies (Hebert and Ward, 1972). Until very recently, ecotoxicity tests were mainly focused on standard endpoints (*e.g.* immobilisation or reproduction) to assess chemicals' effects, though, effects at the subcellular level (*e.g.* gene expression analysis) have had less attention potentially due to the high costs associated. Additionally, cells exposed to chemicals might respond at the level of gene expression to mitigate stress (Borgatta *et al.*, 2015). Genomic tools, such as the DNA microarray technology, can be used to understand molecular mechanisms of toxicity in organisms (Vandegehuchte *et al.*, 2010a). For *D. magna*, a standard species used in ecotoxicology, several microarrays have been developed and used to test the effects of compounds, by assessing transcriptomic changes (Soetaert *et al.*, 2007a; Vandenbrouck *et al.*, 2011). Although the *D. magna* genome is not completely sequenced, the *Daphnia pulex* genome is, which helps identifying genes of interest in *D. magna* (Colbourne *et al.*, 2011).

Considering that contamination in aquatic environments might persist in time and that epigenetic marks might accumulate and lead to changes in the phenotype in subsequent generations, studies on gene expression in a multigenerational approach can

provide insights on long-term exposures, under lower concentrations, which will also provide ecologically relevant approaches (Vandegehuchte *et al.*, 2010a).

The aim of the present study was to assess the effects of a long-term exposure of carbendazim in *D. magna* at the gene transcription level, using a multigenerational experiment (F0-F12). Therefore, two questions can be highlighted as: *i)* what changes are induced by carbendazim in the *D. magna* transcriptome and *ii)* if this changes are kept in time with continuous exposure.

2. Materials and methods

2.1 Test organism

D. magna Straus clone K6 (originally from Antwerp, Belgium) were obtained from continuous cultures maintained in a laboratory at the University of Aveiro (Portugal) and cultured in American Society for Testing and Materials (ASTM) moderated-hard-water medium (ASTM, 1980), with a temperature between 20 ± 1^{0}C and a 16h light-8h dark photoperiod. The medium was renewed three times a week and daphnids were fed with *Raphidocelis subcapitata* (formerly known as *Pseudokirchneriella subcapitata*) at a concentration of $3x10^{5}$ cells/mL and supplemented with an organic extract (Marinure seaweed extract, supplied by Glenside Organics Ltd.).

2.2 Test chemical

Stock solution for carbendazim (CAS No. 10605-21-7, 99.4% purity, Bayer) were prepared in ASTM and then used for preparing the exposure treatments. Chemical analyses were performed to control concentrations of carbendazim in the test medium at Marchwood Scientific Services, Southampton, UK. The analyses for carbendazim were performed by Liquid Chromatography-Mass Spectrometry (LCMS-MS) using the Querchers method. A representative portion of the sample (200-300 mL) was extracted with 20 mL of acetonitrile (containing 1% acetic acid). This was followed by a partitioning step with magnesium sulphate and a subsequent buffering step with sodium acetate. After mixing an aliquot with methanol, the extract was injected directly into the LCMS-MS system (instrument Agilent 6410 Triple Quad LCMs-MS) without any clean-up. A 10 µl

injection volume was utilized. Standards were prepared in solvents at seven levels with recoveries in the range of 70-120%. To determine chemical decay in time, the degradation constant (*k0*) was calculated by the following equation:

$$C_t = C_0 e^{-k0t}$$

Where C_0 corresponds to the initial external concentration (µg/L), K_0 corresponds to the constant of degradation of the chemical in the medium (/hour) and *t* corresponds to time (hours) (Widianarko and Van Straalen, 1996).

2.3 Multigenerational experimental setup

Preliminary toxicity tests were conducted to determine the toxicity of carbendazim to *D. magna* using several endpoints (Chapter 2 - Silva *et al.*, 2015). Based on the reproduction test results, daphnids were exposed throughout the twelve generations to the no observed effect concentration (NOEC) value of carbendazim, corresponding to 5 µg/L (Chapter 2 - Silva *et al.*, 2015). The experimental design is described in Figure 6.1.

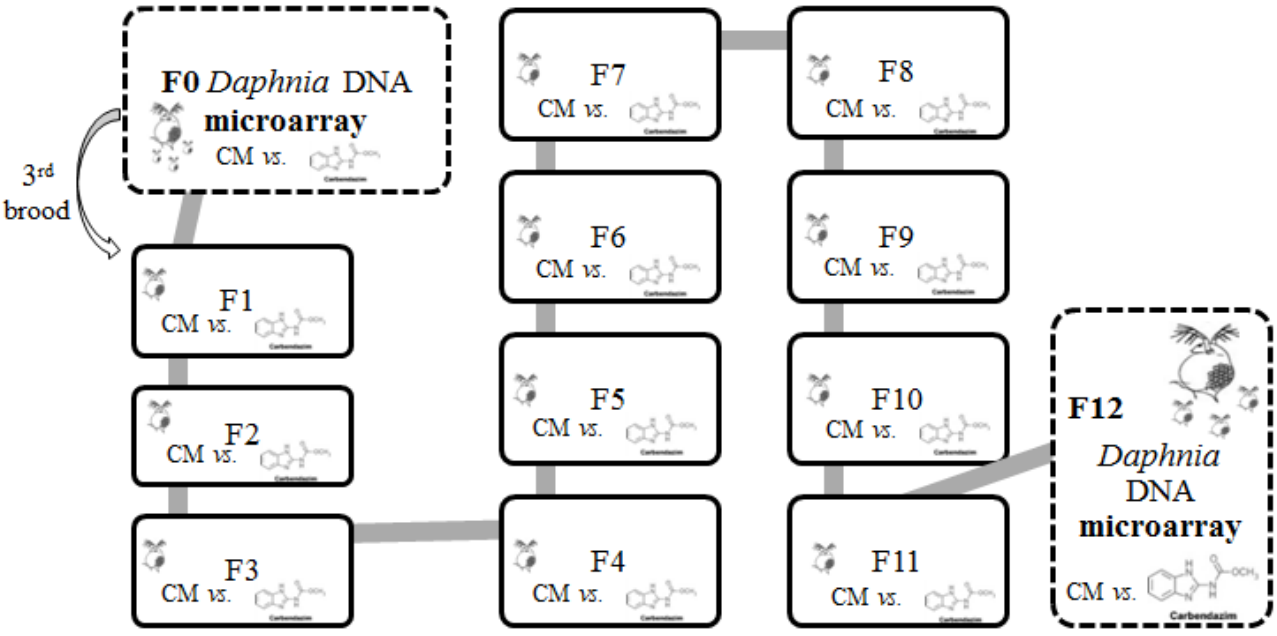


Figure 6.1. Experimental design of the multigenerational test from F0 to F12 generation of daphnids in clean medium (CM) versus exposed to carbendazim.

During the multigenerational experiment, *D. magna* isoclonal populations consisted on three replicates of 1 L glass vessel containing 1L of culture medium and 20 daphnids, for both clean medium and carbendazim (5 µg/L) exposures. Each replicate consisted in ASTM medium with *R. subcapitata* (concentration of $3x10^5$ cells/mL), and was supplemented with an organic extract; the medium was renewed three times a week. *D. magna* neonates of the third brood of the previous generation were used to start the next generation under the same conditions, consisting therefore in a continuous exposure trail. Daphnids kept throughout generations in control conditions (ASTM, *R. subcapitata* and organic extract) will be designated in the paper as daphnids in clean medium and daphnids that were exposed to carbendazim (ASTM, *R. subcapitata*, organic extract and carbendazim) designated as daphnids in carbendazim.

Daphnids' sensitivity was tested with potassium dichromate in F0 and F12 generation, as advised by the OECD 202 guidance protocol (OECD, 2004).

2.3.1 RNA extraction

For the microarray experiment, neonates with less than 24h were picked from the F0 and F12 generations from both clean medium and carbendazim. Three replicates per each treatment were used and consisted of 5 daphnids maintained in the same condition (either in clean medium or carbendazim) until reach 10 days old. Organisms were collected, shock-frozen with liquid nitrogen and stored at -80ºC until RNA extractions were performed.

RNA was extracted using the Trizol® method followed by a column purification step using the RNAeasy kit® and stored at -80°C. Prior to the storage, the purity of the RNA samples (A260/280 and A260/230 ratios) was analysed using Nanodrop® 2000c spectrophotometer (Nanodrop Technologies, USA) and checked for integrity in an Agilent 2100 Bioanalyzer (Agilent Technologies).

2.3.2 Gene expression microarrays

Following RNA extraction, samples were prepared for labeling and hybridization on the microarray. The custom *D. magna* microarrays used in the present work were manufactured for EcoArray by Agilent Technologies, Inc (8 individual arrays per slide)

and was composed by 7370 probes. A total of three replicates per treatment were used and each biological replicate was individually hybridized on the array. A single-colour design was used, using the Agilent one-colour RNA Spike-In Kit (AgilentTechnologies, Santa Clara, CA, USA), following the manufacturer's protocol. Samples were hybridized during 17h at 65ºC with a rotation of 10 rpm and then the microarrays were washed using Agilent Gene Expression Wash Buffer Kit (Agilent Technologies, Palo Alto, CA, USA) according to the manufacturer's protocol.

2.3.3 Microarray data extraction and analysis

Agilent DNA microarray scanner G2505B (Agilent Technologies) was used for scanning. Probes signal values were extracted from microarray scan data using Agilent Feature Extraction Software (Agilent). Data was median normalized using BrB-Array tools v4.4.1 software. Differentially expressed genes in F0 and F12 generations were identified using the Multiple Experiment Viewer Mev software v4.9. An unpaired t-test ($p < 0.05$) with standard Bonferroni correction was performed to identify genes that demonstrated statistically significant differences in expression between clean medium and carbendazim exposed daphnids for both F0 and F12 generations; in addition, differences between F0 and F12 daphnids in clean medium and between F0 and F12 of daphnids in carbendazim were also assessed using the same methodology. Genes were considered up-regulated when the fold change was higher than 1.5 and were considered down-regulated when the fold change was lower than -1.5.

Blast2GO (Conesa *et al.*, 2005) was used to blast and annotate the sequences. To identify GO terms for the identified deregulated genes, the PANTHER (www.pantherdb.org/) tool was used. Although the genome of *D. pulex* is completely sequenced its level of annotation was not sufficient for a complete analysis of gene expression in *D. magna.* Therefore, additional information about putative function of the studied genes was searched using the National Center for Biotechnology Information (NCBI).

2.3.4 Microarray data submission

The microarray raw data have been submitted to Gene Expression Omnibus database and have been given the following accession number: GSE78120.

3. Results and Discussion

3.1 Chemical analyses

To characterize the exposures throughout generations, chemical analyses were carried out within the 48h period of the media renewal. Results showed that carbendazim concentration decreased over time, with a decay rate (*K0*) of 0.03/hour (SE=0.005) (as also described in Chapter 2- Silva *et al.* 2015).

3.2 Multigenerational responses

Changes in organisms' gene expression might be important to understand chemical effects and also to predict future implications at the population level. To our knowledge, the effects of carbendazim on gene expression were never studied in *D. magna*, although similar studies have been carried out with the oligochaeta *Enchytraeus albidus* (Novais *et al.*, 2012). In addition, multigenerational approaches or long-term exposures are not often used to depict effects at the transcriptome level.

In the present study the molecular impact of carbendazim in *D. magna* was investigated under a multigenerational approach and gene transcription results were linked, when possible, with biochemical and individual results obtained in previous experiments.

Considering the results in the microarray experiment, in the hierarchical clustering an evident division was witnessed between samples from the clean medium (Ctrl) and samples from carbendazim (CBZ) for both F0 and F12 generations (Fig. 6.2). The division between samples from clean medium in F0 and F12 was also clear, and the same was verified between samples from carbendazim in both generations (Fig. 6.3). Throughout this study it should be noted that the differential transcription results will be related to the clean medium (control) of the same generation. Gene expression between F0 and F12 generations will be compared in order to analyse differential or similar transcription profiles between generations. Genes that significantly varied in transcription of F0 and F12

in clean medium were removed from the list of differentially transcribed genes of F0 *vs.* F12 with carbendazim, in order to isolate the effects of carbendazim in our results.

The results showed different transcriptional profiles between daphnids from the clean medium and daphnids from carbendazim. The complete list of up and down-regulated genes for generation F0 and F12 is presented in supplementary data (Table 6.1 SD). A decrease in the number of differentially transcribed genes was observed from generation F0 to F12 for daphnids exposed to carbendazim (Table 6.1SD). The list of up and down-regulated genes for F0 *vs.* F12 in clean medium and F0 *vs.* F12 in carbendazim is also presented as supplementary data (Table 6.2SD).

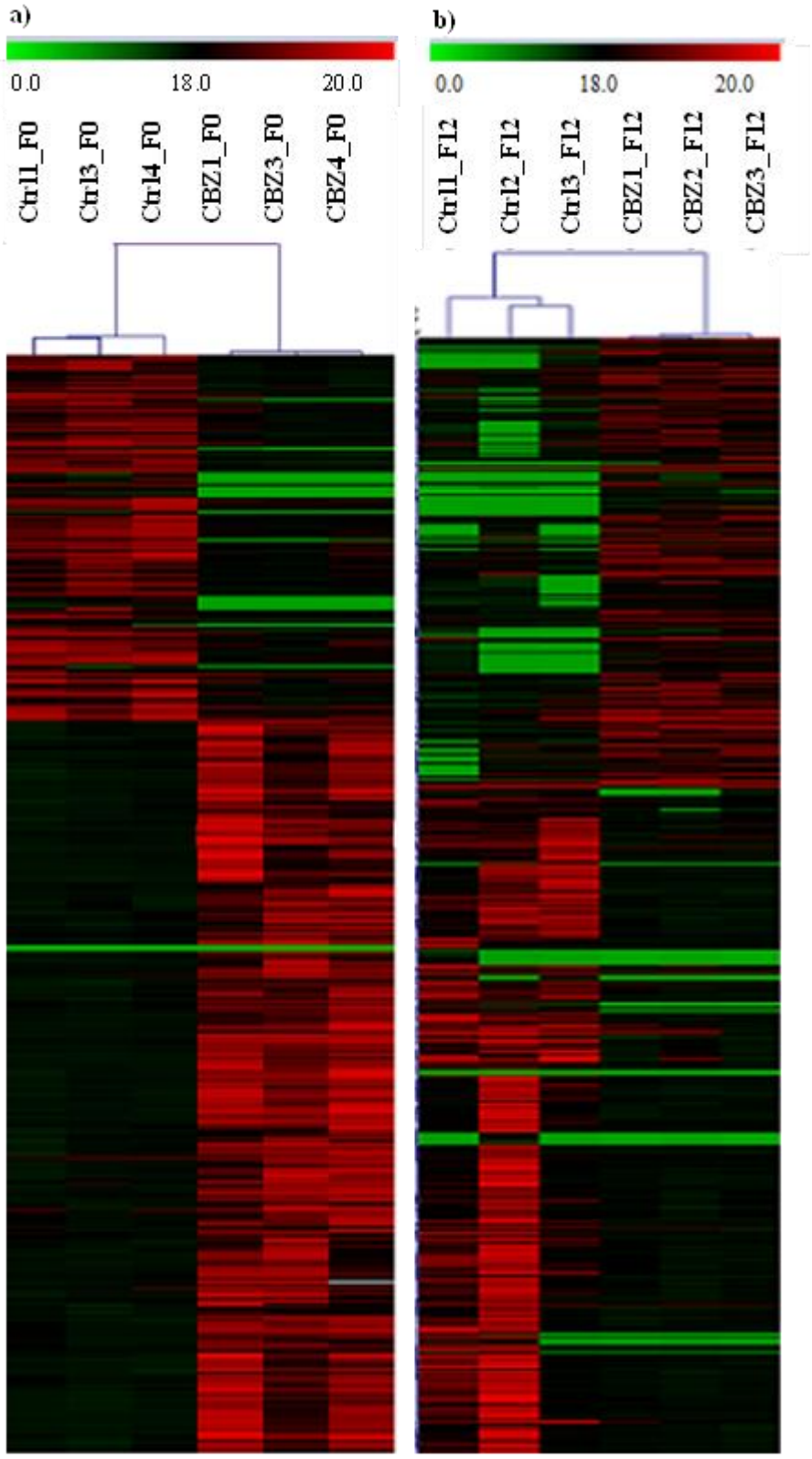


Figure 6.2. Heat map and hierarchical clustering of statistical significant genes of *Daphnia magna* exposed to carbendazim (CBZ) comparing with clean medium (Ctrl) **a)** F0 generation **b)** F12 generation**.** Up-regulation of transcripts is represented by shades of red, and down-regulation by shades of green. Black boxes indicate no significant differential gene expression.

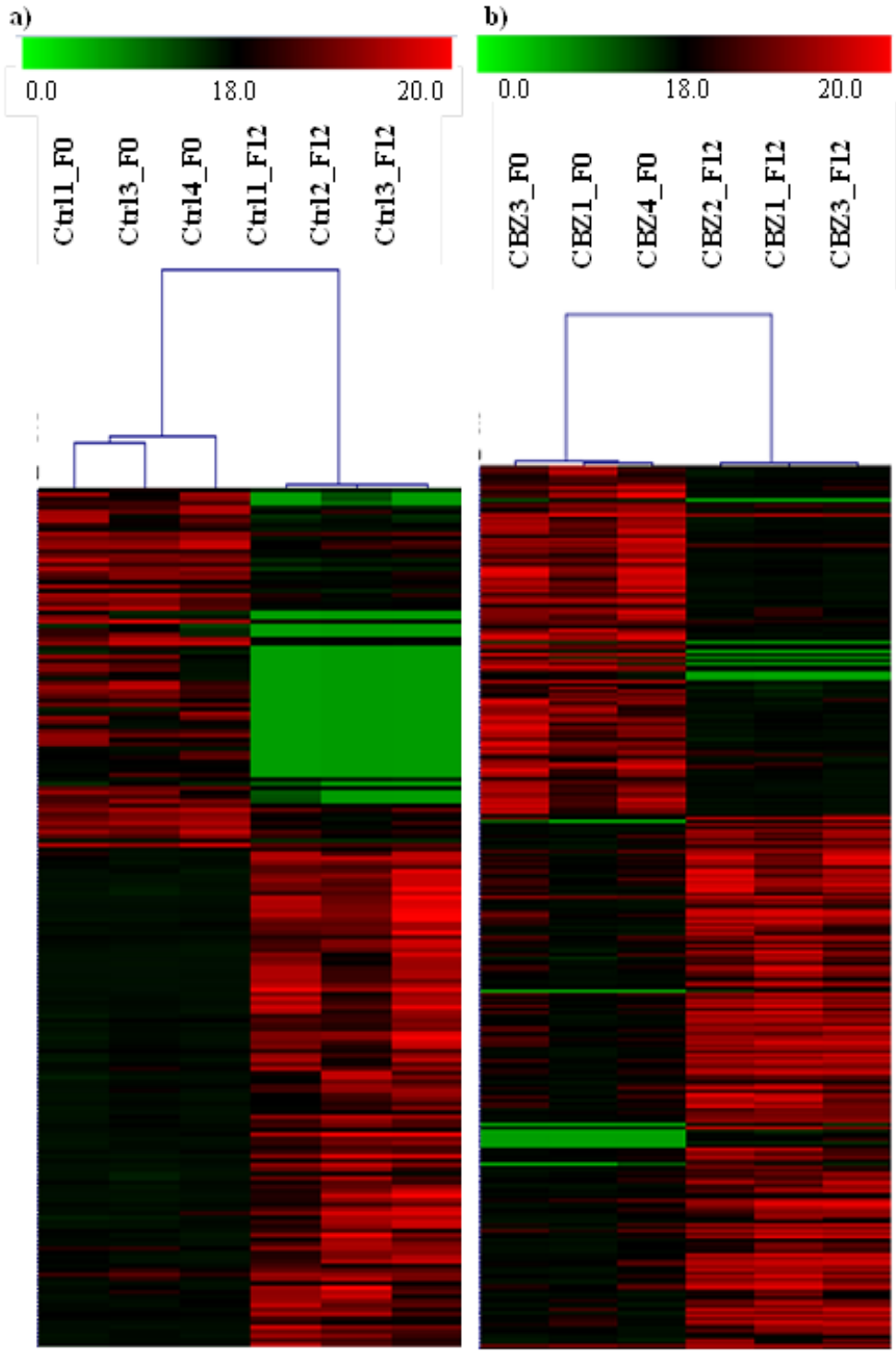


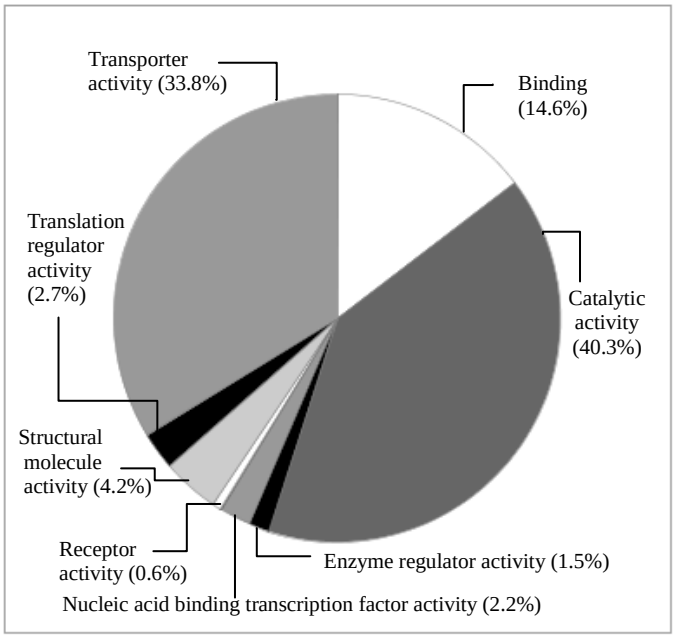
Figure 6.3. Heat map and hierarchical clustering of statistical significant genes in different generations **a)** F0 *vs*. F12 clean medium (Ctrl) **b)** F0 *vs*. F12 carbendazim (CBZ). Up-regulation of transcripts is represented by shades of red, and down-regulation by shades of green. Black boxes indicate no significant differential gene expression.

3.2.1 Gene expression in F0 generation

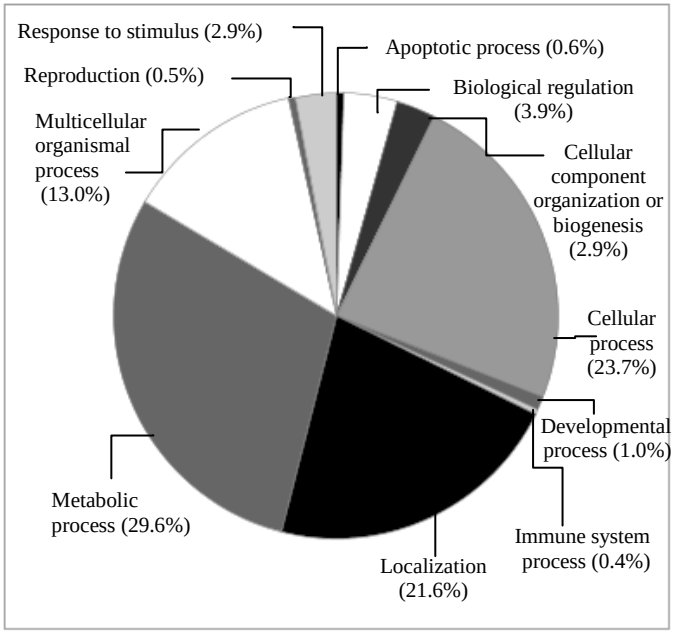
The analysis between daphnids exposed and non-exposed to carbendazim resulted in 191 up-regulated and 98 down-regulated genes (Table 6.1SD). The functional categories of these genes were identified using the PANTHER tool and are shown in Fig. 6.4 and 6.5. The results revealed that in the F0 generation carbendazim caused changes on genes involved in molecular function like catalytic activity, transporter activity and binding (Fig. 6.4a and b).The most affected categories for biological processes were metabolic process, followed by cellular process, localization, and multicellular organismal process (Fig. 6.4a1 and b).

The effect of carbendazim at the gene expression level on *D. magna* was noticed early on (F0). The gene encoding for histone deacetylase Rpd3 (*HDAC Rpd3*) (*Tribolium castaneum*) was up-regulated 14.83-fold (Table 6.1SD). This protein, among other functions, contributes to maintenance of DNA integrity, since DNA is wrapped around histones, and acetylation and de-acetylation influences DNA expression. HDAC is responsible for the removal of acetyl groups, increasing ionic interactions (between the positively charged histones and negatively charged DNA), which produces a more compact chromatin structure and represses gene transcription and therefore is associated with gene silencing (Ropero and Esteller, 2007). Meanwhile, the coding gene for the DNA polymerase epsilon catalytic subunit A (*Plasmodium falciparum*) was found -2.21-fold down-regulated, as well as a gene encoding for a DNA mismatch repair protein (*Xanthomonas campestris*) (-2.63-fold). These results appear to be supported by our previous experiments where an increase in DNA damage (as DNA strand breaks evaluated using the comet assay) was observed after 24h exposure of *D. magna* to carbendazim (Chapter 2 - Silva *et al.*, 2015). In this generation a 6.98-fold transcriptional induction of a homologous gene coding for a ubiquitin-conjugating enzyme E2 variant 2-like protein (*UBE2V2*) (*Apis mellifera*) and 4.44-fold for an excinuclease ABC, subunit A (*uvrA*) (*Halothermothrix orenii*) occurred. Novais *et al.* (2012) also verified that DNA damage and repair processes were significantly affected after the exposure of *E. albidus* to carbendazim. Genes coding for intermediate filament proteins involved in DNA repair were significantly induced at all concentrations, supporting the genotoxic effects of carbendazim.

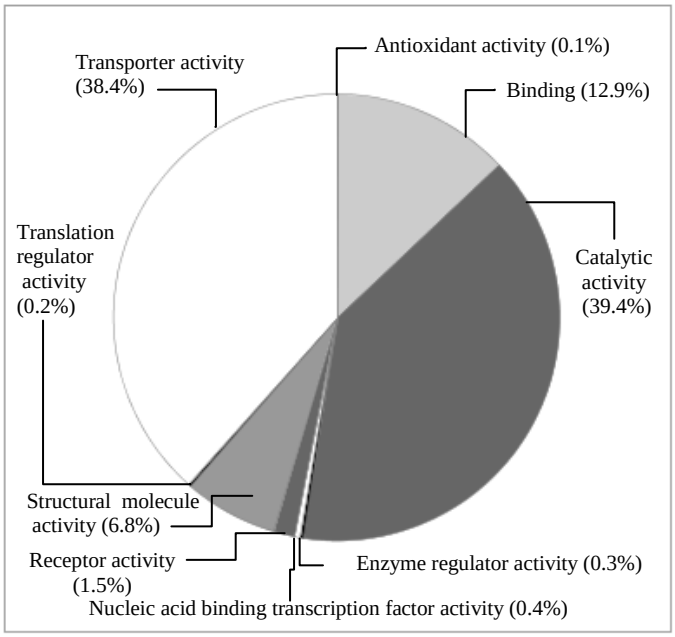
a) F0 up-regulated genes, Molecular Function



a1) F0 up-regulated gene, Biological Process



b) F0 down-regulated genes, Molecular Function



b1) F0 down-regulated gene, Biological Process

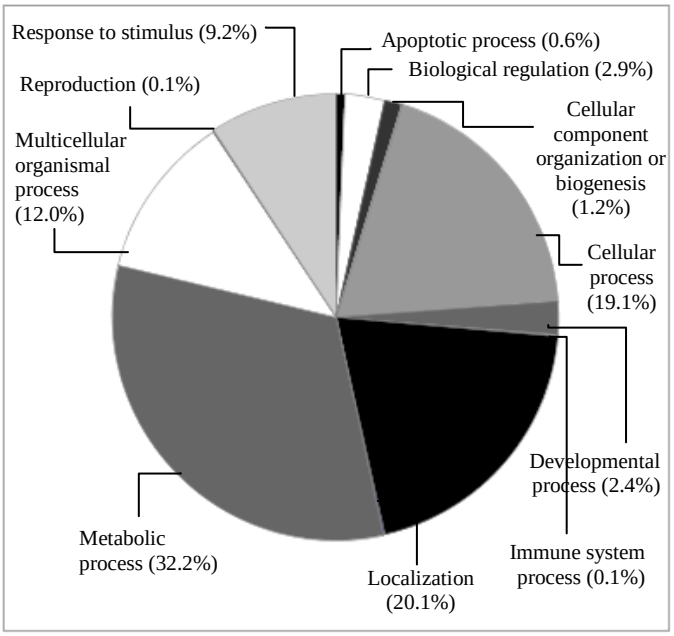


Figure 6.4. Differentially expressed genes were classified in functional categories using PANTHER **a)** F0 generation, up-regulated genes, molecular function **a1)** F0 generation, up-regulated genes, biological process **b)** F0 generation, down-regulated genes, molecular function **b1)** F0 generation, down-regulated genes, biological process; the percentage of affected genes is presented between brackets.

The gene coding for papilin-like protein (*Apis mellifera*) was 3.41-fold up-regulated as well, these genes are considered serine-type endopeptidase inhibitors, preventing or reducing the activity of serine-type endopeptidases. A 5.02-fold up-regulation of glutathione *S*-transferase T1 (*GSTT1*) (*Apis mellifera*) homologues was observed. GST plays an important role in biotransformation/cellular detoxification processes of various chemicals and defence against peroxidative products of DNA (Henson *et al.*, 2001; Hyne and Maher, 2003). Some biochemicals parameters, including GST activity, were previously evaluated in a similar multigenerational experiment, being observed an increase in the activity of this enzyme in F0 carbendazim exposed daphnids comparing with the F0 clean medium (Chapter 5). The transcriptionally up-regulation of genes related with anti-oxidant activities indicate a possible response to oxidative stress (Livingstone, 2003). Indeed, David and collegues (2011) observed that generally *D. magna* exposed to genotoxicants had an up-regulation in the genes encoding GST.

The chromobox homolog 1 (*CBX1*) (*Xenopus tropicalis*) gene was -2.77-fold down-regulated. This encoding gene is a component of heterochromatin that might contribute to the association of the inner nuclear membrane with the heterochromatin (Wreggett *et al.*, 1994). This gene binds histone H3 tails methylated at "Lys-9", contributing to epigenetic repression. Jointly with *CBX1*, Methylmalonyl-CoA epimerase (*MCEE*) (*Macaca* mulatta) was also -4.75-fold down-regulated, a gene involved in the catabolism of fatty acids. *Sec16* isoform F (*Yarrowia lipolytica*) gene was considerably down-regulated (13979.40-fold) upon exposure to carbendazim and it is involved in the *in vivo* transport between the endoplasmic reticulum and the Golgi apparatus (Bharucha *et al.*, 2013). *Daphnia* have small Golgi complexes that are involved in the flow of secretory proteins from the endoplasmic reticulum to the cell exterior (Elser *et al.*, 1996; Zaffagnini and Zeni, 2009). Golgi complexes are also involved in the lipid transport, which showed to be affected in the present study. Other genes involved in the Golgi apparatus/vesicle transport were affected in carbendazim exposed F0 generation, including two SNARE genes (a homologous protein of the Golgi SNAP receptor complex member 1 (GOSR1) in *Strongylocentrotus purpuratus* and a vesicle transport protein SEC20 homologous in *Tribolium castaneum*) and an AP-1 complex subunit sigma-2 (*Tribolium castaneum*), which were 8.93, 11.42 and 10.55-fold up-regulated, respectively.

The gene coding for a protein homologous to Gaba(A) receptor associated protein (*Branchiostoma belcheri tsingtaunese*) was -2.83-fold down-regulated; this protein mediates inhibitory neurotransmission (Kittler and Moss, 2003). Likewise, there was a 9.41-fold up-regulation of the encoding gene *slowmo* (*Bombyx mori*), known to cause inhibition of neurotransmitter release (Carhan *et al.*, 2003). The coding gene of glucosamine 6-phosphate N-acetyltransferase (*GNPNAT1*) (*Anopheles gambiae*) that participates in the glutamate metabolism was -3.75-fold repressed. In the study of Vandegehuchte *et al.* (2010b) the *GNPNAT1* gene was up-regulated in *D. magna* after zinc exposure (F0). Glutamate is an excitatory neurotransmitter that plays a principal role on neural activation (Fonnum, 1984), which could suggest that carbendazim might cause adverse neural effects on *D. magna*. However, there are no results of previous experiments to support this hypothesis.

Several transcripts involved in the electron transport chain system and oxidative phosphorylation were affected: up-regulation of genes encoding for ATP synthase, cytochrome b (*cytb*) (5.76-fold), cytochrome c1 (*cytc1*) (7.97-fold) and cytochrome oxidase subunit II (*COII*) (5.71-fold), cytochrome c oxidase (*cox*) (4.55-fold), and down-regulation of NADH dehydrogenase I (-20664.18-fold) and cytochrome b-c1 complex (*cyt b/c1*) subunit 2 (-3.79-fold). These results suggest that carbendazim might affect the mitochondrial activity and consequently the ATP production (Novais *et al.*, 2012). Novais *et al.* (2012) also observed the impact of carbendazim on mitochondrial genes expression on the worm *E. albidus* after a 2 days exposure. The induction (6.87-fold) of the homologous gene coding for the solute carrier family 25 (mitochondrial thiamine pyrophosphate carrier) member 19 (*SLC25A19*) (*Bos Taurus*) was verified as well. An induction of ATP synthase was also observed when *D. magna* was exposed to propiconazole. The authors suggested that this might be related to higher requests of energy (as ATP) to cope with stress/detoxification (Soetaert *et al.*, 2006). This might also enlighten the reduction in the number of neonates produced by the F0 daphnids exposed to carbendazim (comparing to the F0 daphnids in clean medium) in a similar exposure (Chapter 5).

A transcriptional up-regulation (11.59-fold) of the homologous gene of the cytochrome P450 (*CYP450*) (*Homarus Americanus*) was observed. Accordingly, the induction of a *CYP450* was also found in *D. magna* exposed for 24h to cadmium by

Connon *et al.* (2008), as well as for the study by David *et al.* (2011), where the up-regulation of P450 genes is related to higher xenobiotic and metabolism capacity of daphnids. CYP450 proteins are known to be involved in lipid metabolism (*e.g.* fatty acids), hormone synthesis/breakdown and phase I detoxification systems (Baldwin *et al.*, 2009; Vandegehuchte *et al.*, 2010c).

Homologous genes for proteins related to several metabolic pathways were affected as well. In F0 generation, up and down transcription of genes involved in lipid metabolism were observed: up-regulation of glycerol-3-phosphate dehydrogenase (*GPDH*) (9.74-fold), hydroxysteroid dehydrogenase like 2 (*hsdl2*) (12.00-fold) and fatty acid desaturase (*FADS*) (8.57-fold); down-regulation of hydroxyacyl dehydrogenase subunit A isoform 3 (-2.91-fold), pyrroloquinoline-quinone aldehyde dehydrogenase (-3.49-fold) and *MCEE*. Lipids are known to be specifically involved in egg production in cladocerans (Goulden and Henry, 1987). The transcriptional deregulation of genes involved in the lipid metabolism might explain the reduction in the number of neonates in F0 carbendazim exposed *D. magna* (comparing with clean medium) (Ribeiro *et al.*, 2011). In addition, the carbohydrate metabolism was also affected, with up-regulated genes being also expressed: polypeptide N-acetylgalactosaminyltransferase 5 (*GALNT5*) (18.77-fold), UDP-glucosyl transferase family protein (13.07-fold), multifunctional fatty acid oxidation complex subunit alpha (fadJ) (6.24-fold).

Several genes encoding ribosomal proteins were repressed, including the ribosomal protein S1a (*RPS1a*) (-2.10-fold), ribosomal protein S27 (*RPS27*) (-3.49-fold) and 39S ribosomal protein L44 (*RPL44*) (-3.68-fold). This suggests that carbendazim might cause impairment in translation processes. Vandegehuchte *et al.* (2010c) also observed a down-regulation of ribosomal proteins for the F0 generation exposed to zinc. The authors related this down-regulation with a potential energy-saving mechanism after the stress induced by the compound. These authors also reported that this transcriptional repression was not present in the next generation. Similarly, some ribosomal proteins were found repressed when *D. magna* was exposed to the fungicide fenarimol (Soetaert *et al.*, 2007a), as well as alterations in the expression of several ribosomal proteins, after the exposure of *D. magna* to the fungicide propiconazole (Soetaert *et al.*, 2006).

The heat-shock protein 70 (*HSP 70*) (*Entamoeba histolytica*) was transcriptionally repressed (-3.47-fold). These proteins are important as ATP-dependent molecular

chaperones, helping in polypeptides folding and targeting of proteins for lysosomal degradation (Rohde *et al.*, 2005). Other central roles of HSP 70 include response to cellular stress (Morano, 2007) and block of apoptosis through binding a protease activating factor-1 and therefore preventing the constitution of the apoptosome (Ravagnan *et al.*, 2001). The down-regulation of *HSP70* probably slows/hampers the cellular recovery (Rohde *et al.*, 2005). Other genes involved in apoptosis were deregulated, like a gene coding for an apoptosis-resistant E3 ubiquitin protein ligase 1 (*AREL1*) (*Tribolium castaneum*) that was -116578.52-fold down-regulated and the homologous gene encoding for growth hormone-inducible transmembrane (*GHITM*) protein-like (*Apis mellifera*) which was 11.75-fold up-regulated.

Two genes involved in embryogenesis were affected (Gu *et al.*, 1999): a homologous gene coding for the oocyte maturation factor Mos (*Anas poecilorhyncha*) was found up-regulated and a homologous *MAST1* gene (*Rattus norvegicus*) was -3.03-fold repressed. Exposure to carbendazim has been referred as interfering with microtubules assembly, causing a gradual disappearance of microtubules (Davidse, 1986). Novais *et al.* (2012) observed a down-regulation of the encoding gene for Stathmin 1 oncoprotein 18 which are directly involved in microtubule assembly/disassembly (Takahashi *et al.*, 2002). In several studies, carbendazim also caused a decrease in the number of *D. magna* neonates and an increase in the number of aborted eggs with increasing concentrations of carbendazim, which is possibly related with mitosis inhibition during the eggs division in the brood pouch (Canton, 1976; Ribeiro *et al.*, 2011; Chapter 2 - Silva *et al.*, 2015).

3.2.2 Gene expression in F12 generation

A lower number of differentially transcribed genes was observed for the F12 generation comparing with the F0: 53 genes were up and 66 were down-regulated (Table 6.1SD). Vandegehuchte *et al.* (2010c) also verified a decrease of differentially expressed transcripts throughout three generations of *D. magna* exposed to zinc. Regarding individual endpoints, results from zinc exposures lead to negative effects on body length and reproduction at the second generation when compared with the control. However, in the third generation no differences were observed between treatments and control, suggesting that the organisms of this generation could be acclimated to zinc (Vandegehuchte *et al.*, 2010c). In the F12 generation of daphnids in carbendazim, a lower

number of processes were affected along with the decrease in the number of up and down-regulated genes when compared to the F0. Carbendazim caused changes in the gene expression of proteins involved in some molecular functions, causing changes mainly on transporter, catalytic and binding activity (Fig. 6.5a and b). Regarding biological processes, the categories with more affected genes were localization, metabolic process, cellular process and multicellular organismal process (Fig. 6.5a1 and b1).

The gene coding for the homologue protein testican-1-like in *Apis mellifera* was also 1.95-fold induced (Table 6.1 SD), which is a protein involved in several neural mechanisms at the central nervous system (Takahata *et al.*, 2010).

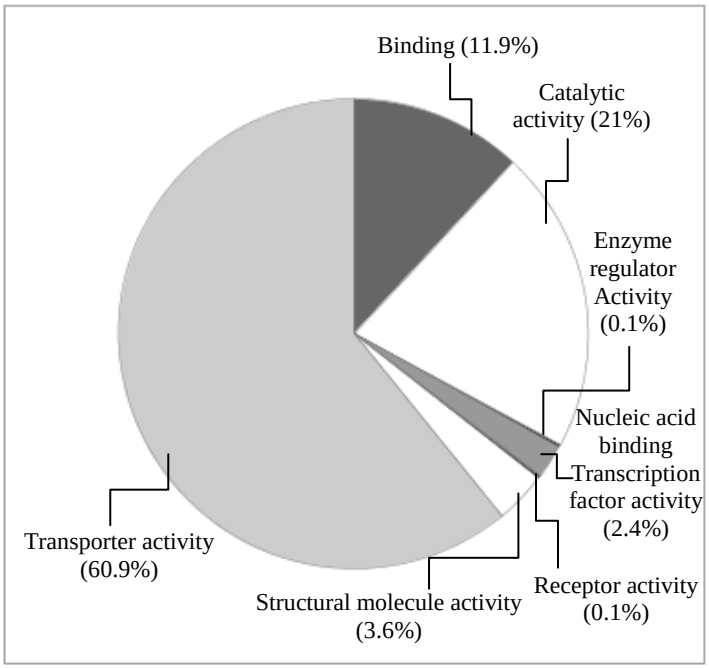
In this generation genes involved in ATP production were also affected, namely the NADH dehydrogenase subunit 6 (*ND6*) (*Mustelus manazo*), which was 15117.52-fold up-regulated, and the *cytb*, was found to be -6.30-fold repressed.

The 39S *RPL44* (*Tribolium castaneum*) was 2.25-fold up-regulated as well as the asparagine-tRNA ligase putative (*Plasmodium falciparum*) 7605.56-fold, which is involved in the protein biosynthesis. The ribosomal protein L5 (*RPL5*) (*Bombyx mori*) was -7.93-fold down-regulated. Additionally, it was observed a down-regulation of genes encoding for proteins with homology to a novel protein similar to vertebrate topoisomerase (DNA) II beta 180kDa (*TOP2B*) of *Danio rerio* (-3.25-fold) and a *UBE2V2*-like (*Apis mellifera*) (-9.26-fold). The DNA polymerase epsilon catalytic subunit A, which was found to be down-regulated in F0, was 1.72-fold up-regulated in F12.

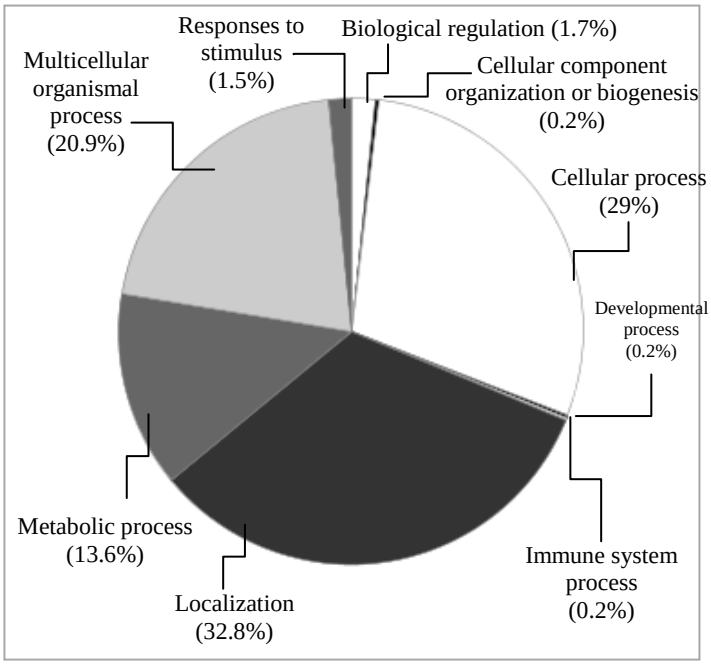
The double stranded RNA-activated protein kinase 1 (*PKR1*) (*Tetraodon nigroviridis*), responsible for apoptosis induction (Takizawa *et al.*, 2002), was also found 35149.50-fold up-regulated. The *fem-1* homolog B (*Tribolium castaneum*) was -8.41-fold repressed, which encodes a protein that belongs to the death receptor-associated family of proteins and therefore is also implicated in apoptosis (Chan *et al.*, 1999). In addition, this protein is also involved in the regulation of DNA damage checkpoint and consequently, its repression might have consequences on DNA damage. Jiang *et al.* (2014) studied the effects of carbendazim in the zebrafish *D. rerio* and identified differential transcription of genes playing a critical role in cell apoptosis pathways. Considering that some genes related to apoptosis were affected upon carbendazim exposure, further investigations

should be performed to understand how this genes are influencing the process of apoptosis in *D. magna*.

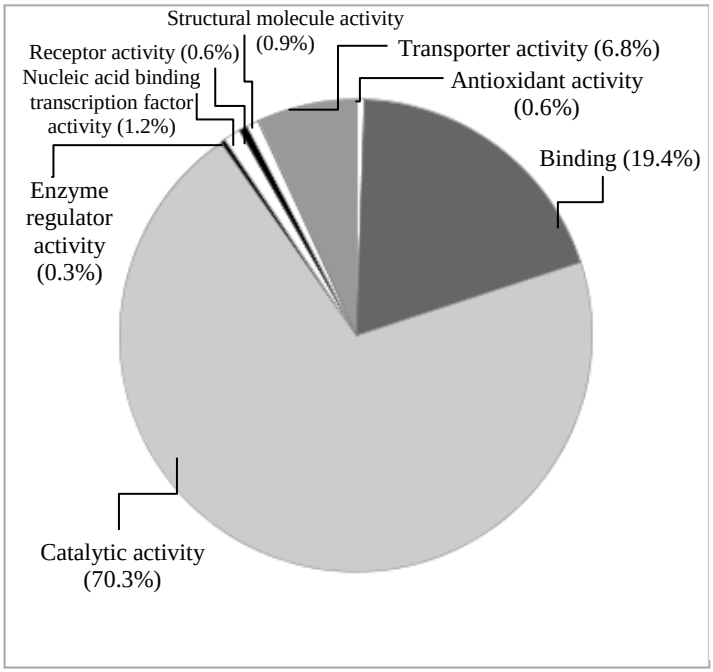
a) F12 up-regulated genes, Molecular Function



a1) F12 up-regulated gene, Biological Process



b) F12 down-regulated genes, Molecular Function



b1) F12 down-regulated gene, Biological Process

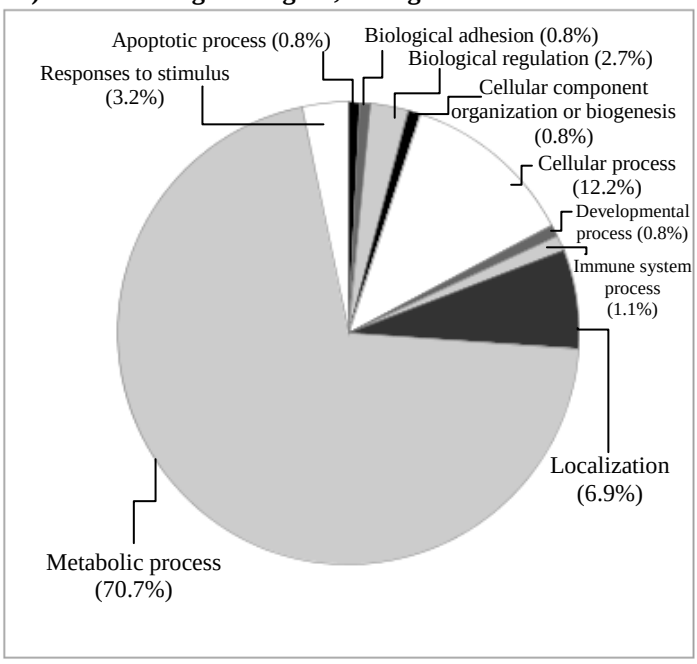


Figure 6.5. Differentially expressed genes were classified in functional categories using PANTHER **a)** F12 generation, up-regulated genes, molecular function **a1)** F12 generation, up-regulated genes, biological process **b)** F12 generation, down-regulated genes, molecular function **b1)** F12 generation, down-regulated genes, biological process; the percentage of affected genes is presented between brackets.

Genes involved in embryonic development were deregulated, with the (18679.08-fold) up-regulation of the coding gene for a protein with homology to Pax-6 protein in *Euprymna scolopes* (Kannan and Vincent, 2015) and the repression (-5.79-fold) of the gene encoding for the protein bicaudal C (*Tribolium castaneum*) (Tran *et al.*, 2010). Carbendazim acts on cell division, inhibiting the development of the germ tubes in the nucleus and inhibiting the reproduction capacity in *D. magna* (Davidse, 1986; Ribeiro *et al.*, 2011; Chapter 2 - Silva *et al.*, 2015). Therefore, it is expected that genes belonging to these processes are differentially expressed upon exposure to carbendazim.

One homologous gene of a di-domain hemoglobin precursor (*Daphnia pulex*), a gene involved in oxygen transport, was -7.34-fold repressed after the long-term exposure reflected by F12. Soetaert *et al.* (2007b) observed the same trend after 48h of cadmium exposure in *D. magna*. The author suggested that the down-regulation of genes involved in this process could indicate a reduction on the oxygen levels transported. Martinez-Tabche and her team (2000) observed a decrease of the hemoglobin levels when exposing the worm *Limnodrilus hoffmeisteri* to zinc, suggesting that the inhibition of heme synthesis is induced by this metal. Vandegehuchte *et al.* (2010) have hypothesized that if the transcription of coding genes of hemoglobin are down-regulated, this could indicate an energy-saving mechanism.

In the present study, after twelve generations the deoxyuridine triphosphatase (*dut*) (*Homo sapiens*), an enzyme of the nucleotide metabolism also involved in DNA replication, was 3.21-fold up-regulated. Curtin *et al.* (1991) reported that an increase in intracellular dut might lead to DNA strand breaks and consequently cell death.

Some differentially expressed genes were common between F0 and F12 generations, sharing five up-regulated genes and two down-regulated genes. Two of the common up-regulated genes were a gene similar to YY1 transcription factor and a gene similar to dispatched homolog 1 (*Disp1*) (Table 6.1). This transcription factor has an important role in biologic processes, including embryogenesis, replication and cellular proliferation, having important properties that initiate suitable cellular development (Gordon *et al.*, 2006). YY1 in turn might activate the p53 (a tumour-suppressor protein) in response to genotoxic stress (Gronroos *et al.*, 2004). Several studies have already demonstrated the genotoxic potential of carbendazim for different species (JanakiDevi *et al.*, 2013; Chapter

2 - Silva *et al.*, 2015; Singh *et al.*, 2008). The *Disp1* is related with organ morphogenesis and hedgehog receptor activity, which is important for proper development in embryonic cells (Caspary *et al.*, 2002).

Table 6.1. Gene ID and description of common **a)** up-regulated and **b)** down-regulated genes in the F0 and F12 generations following *Daphnia magna* exposure to carbendazim.

	Gene ID	***Gene description [species]***
Up-regulated genes	YP_548045	hypothetical protein Bpro_1196 [*Polaromonas* sp.]
	AAY54998	IP06749p [*Drosophila melanogaster*]
	XP_001069615	PREDICTED: similar to YY1 transcription factor [*Rattus norvegicus*]
	AAY66970	secreted protein [*Ixodes scapularis*]
	XP_785823	PREDICTED: similar to dispatched homolog 1 [*Strongylocentrotus purpuratus*]
Down-regulated genes	XP_678020	hypothetical protein [*Plasmodium berghei*]
	CAE73165	hypothetical protein [*Caenorhabditis briggsae*]

3.2.3 Gene expression of F0 vs. F12 in clean medium

Gene transcription patterns of F0 *vs.* F12 daphnids in clean medium were compared and differentially transcribed genes were found (Table 6.2SD). Several ribosomal proteins, including *RPL34* (16.29-fold) and *RPS3a* (4.72-fold) were up-regulated and 50S *RPL2* was repressed (-4.50-fold). Several transcripts involved in the electron transport chain system and oxidative phosphorylation were shown affected: *cytb* (*D. pulex*) (7.32-fold) and *COII* (*Pierris rapae*) (9.14-fold) were up-regulated. Encoding gene for vitellogenin receptor (*Blattella germanica*), a gene involved in oocyte development, was 5.54-fold up-regulated (Dominguez *et al.*, 2014). A gene involved in the maintenance of DNA integrity, *HDAC Rpd3* (*Tribolium castaneum*), was found 4.54-fold up-regulated (Ropero and Esteller, 2007).

As previously stated, in our microarray analysis, genes that significantly varied in transcription between the F0 and F12 clean medium were excluded from the F0 and F12 carbendazim analysis. Variability in gene expression among clean medium generations might provide some insights regarding the changes in responses (in LC_{50} or EC_{50} values) in daphnids kept in the same conditions (*e.g.* medium, temperature, light:dark photoperiod, type of food) and exposed to the same compound within different times. Vandegehuchte *et al.* (2010a, 2010b) reported that a large number of genes were differentially transcribed in

daphnids from control treatments as well. The authors suggested that this difference might be attributed to differences in physiological processes, for instance, in reproductive cycles or in molting phases (Vandegehuchte *et al.* 2010a, 2010b). In the earthworm *Lumbricus rubellus* significant differences in gene expression were also found between two control groups (Owen *et al.*, 2008).

3.2.4 Gene expression of F0 vs. F12 carbendazim

Alterations on gene transcription profiles in daphnids were observed after carbendazim exposure at the end of twelve generations (Table 6.2SD). Results showed the transcriptional induction of genes related with ATP production, namely homologous encoding genes of NADH dehydrogenase I (*Errhomus variabilis*) (13302.91-fold), ATP synthase mitochondrial F1 complex assembly factor 1 (*ATPAF1*) (*Tribolium castaneum*) (3.10-fold) and the *cyt b/c1* subunit 2 (*Tribolium castaneum*) (5.13-fold). Some genes encoding ribosomal proteins were also up-regulated, including the 39S *RPL44* (6.20-fold), *RPS27* (*Bombyx mori*) (6.12-fold), *RPL10Ae* (*Biphyllus lunatus*) (3.25-fold) and *RPS1a* (*Xenopus laevis*) (3.23-fold). A homologous gene coding for an outer membrane lipoprotein *Blc* (*Rhodobacterales bacterium*) was 75647.24-fold induced as well. This protein is expressed under stress conditions and has as the main function to store and carry lipids (Campanacci *et al.*, 2004). It was also verified a notorious transcriptional induction of a homologous gene coding for the protein sec16, isoform F (*Yarrowia lipolytica*) (65357.29-fold) with possible consequences in the protein transport (Bharucha *et al.*, 2013).

The transcriptional up-regulation (8.79-fold) of the coding gene of the tyrosine phosphatase (*Trypanosoma brucei*) was noticed. These proteins are specific regulators of signaling (Tonks, 2013) and are essential in homeostasis in eukaryotic cells. Disturbances on their functions may cause several diseases (Bohmer *et al.*, 2013).

The homologous gene coding for a programmed cell death protein (*PCD*) 6 (*Tribolium castaneum*) was 3.67-fold up-regulated. PCD proteins can establish the equilibrium between survival and death of normal cells. These proteins play an important role on deciding cancer cell fate when this equilibrium suffers disturbances (Ouyang *et al.*, 2012). Gene coding for GEF (*Canis familiaris*) was also 3.58-fold up-regulated, as well as the homologous gene coding for serine/threonine-protein kinase (STKs) vrk (*Aedes*

aegypti) (2.40-fold). The GEF enzyme catalyzes the exchange of guanosine diphosphate (GDP) and guanosine triphosphate (GTP) in a G-protein (Bos *et al.*, 2007).

The expression of the homologous coding gene of a DNA polymerase epsilon catalytic subunit A (*Plasmodium falciparum*), a protein that participates in DNA repair and in chromosomal DNA replication was 3.83-fold transcriptionally induced (Shinbrot *et al.*, 2014). The induction of the homologous gene coding of a protein similar to MCEE occurred as well (*Macaca mulatta*) (4.68-fold) with possible consequences to the fatty acid metabolism and consequently in energy production (Goulden and Place, 1990).

The transcriptional down-regulation of genes is also presented in Table 6.2SD. The homologous gene coding for melanoma antigen family B10 (*MAGEB10*) (*Homo sapiens*) was found -1.57-fold down-regulated. Some melanoma antigen genes are involved in reproduction, apoptotic processes, cell death and are expressed in melanomas (tumors) (Rogner *et al.*, 1995).

Gene expression with homology to a tubulin tyrosine ligase-like protein 2 (*TTLL2*) (*Tribolium castaneum*) was repressed (-1.63-fold). This protein has a relevant role in regulation of microtubule function (Preston *et al.*, 1979).

The serine/arginine-rich splicing factor 11 (*SRSF11*) (*Gallus gallus*) encoding gene was -1.89-fold down-regulated; this gene plays a role in pre-mRNA processing, in splicing and in the control of gene expression (Listerman *et al.*, 2013). A gene involved in oxygen transport, di-domain hemoglobin precursor (*Daphnia pulex*), was -4.61-fold repressed in our study and was also observed in daphnids exposed to cadmium in another study (Soetaert *et al.*, 2007b).

The HMG box protein (*Tetrahymena thermophile*) plays a role in protein-protein interactions, being involved in DNA-binding (Stros *et al.*, 2007), and its corresponding mRNA was found down-regulated (-6.68-fold) after twelve generations of daphnids exposed to carbendazim. A gene involved in embryo development, the oocyte maturation factor Mos (*Anas poecilorhyncha*), was -8.87-fold transcriptional repressed (Brevik *et al.*, 2011). A gene encoding for cyclin K (*Canis familiaris*) was found down-regulated (-6377.84-fold). Cyclins play a role as regulators in cell cycle, specifically cyclin K, which has a role in replication response after stress, neurogenesis and in embryo development (Kohoutek and Blazek, 2012). Effects on gene expression of embryo development related

genes have already been reported in *D. magna* exposed (for 96h) to the fungicide fenarimol (Soetaert *et al.*, 2007a).

Two genes encoding ribosomal proteins were down-regulated: the 40S *RPS3a* (*Tribolium castaneum*) (-7.36-fold) and the ribosomal protein S7 (*RPS7*) (*Saprolegnia ferax*) (-8.85-fold). Studies showed that the inhibition of expression of the *RPS3a* could induce apoptosis in tumor cells (Naora *et al.*, 1998). The significance of ribosomal proteins that were down-regulated has already been discussed previously in this work.

The down-regulation (-17088.07-fold) of histidine kinase (HK) (*Psychrobacter cryohalolentis*) was verified. HK have multiple functions, including an important role in signal transduction (Dutta *et al.*, 1999).

It should be highlighted that the array does not contain the complete genome of *D. magna* and consequently, some results were not deeply investigated, which can be seen as a limitation in the present study. Additionally, the *D. magna* genome is not completely annotated and therefore the results obtained in the present study are based on gene homologies for a vast number of organisms found in different databases. Consequently, these functions might not be exactly the same in *D. magna* and they were merely an indication of the possible gene's function.

Changes in mRNA levels are good indicators of gene regulation, but cannot be used alone to directly correlate with protein expression due to varied post-transcriptional mechanisms that are involved in the synthesis of the native protein. To conclude on the expression of a protein that is encoded by a de-regulated gene, additional studies must be conducted, namely proteomics and metabolomics studies. Although we are not able to extrapolate the mechanism of action of carbendazim solely based on transcriptomics data, we have gathered valuable information regarding the influence of carbendazim at the gene expression level that might be used as biomarkers of exposure.

The present study contributed to unravel alterations at the gene expression level of carbendazim in daphnids using an environmentally relevant concentration. In this long-term exposure to carbendazim few effects at the individual level or transposed to the populations could be depicted throughout generations (till F12). Individual effects were

mainly observed when assessing daphnids longevity, which significantly decreased in F12 carbendazim exposed daphnids, when comparing with F12 daphnids kept in clean medium (Chapter 5). Considering that subcellular effects like the ones depicted in the present study are usually early warning signals, it is still difficult to interpret the (few) individual and population effects observed under the same level of exposures. Li *et al.* (2015) reported that it was not possible to create a direct link between molecular and individual effects after *D. magna* has been exposed to an environmentally relevant concentration of the synthetic flame retardant tris (1,3-dichloro-2-propyl) phosphate. Notwithstanding, the deregulated genes obtained at 5 μg/L in the present study are related to effects at the individual and population levels under higher exposure concentrations. At the individual level, the lowest concentration where effects were observed in terms of neonates' production or aborted eggs were 20 and 35 μg/L, respectively (Chapter 2 - Silva *et al.*, 2015).

4. Conclusions

In this study, carbendazim was shown to cause changes at the gene expression level in F0 and in F12 generations of *D. magna*. The highlights from this study can be summarized as follows: *i*) carbendazim induced changes in genes involved in response to stress, DNA replication/repair, embryogenesis, neurotransmission, protein biosynthesis, ATP production, lipid and carbohydrate metabolism; *ii)* these changes were not kept in time, since a lower number of differentially transcribed genes was observed after twelve generations of daphnids exposed to carbendazim and the pathways were differentially affected in the F0 and F12. Only five up-regulated genes and two down-regulated genes were common between F0 and F12. Overall, the common up-regulated genes were involved in embryogenesis, cellular proliferation and replication. In particular, the transcriptional factor YY1 can be highlighted as a responses related to genotoxic stress. Therefore, the transcriptome results from the present study seem to support the genotoxic effects of carbendazim previously described using the comet assay and the increase of aborted eggs with decrease of number of neonates produced (Chapter 2 - Silva *et al.*, 2015).

Considering that the *D. magna* genome is not completely sequenced and some genes were un-annotated, it would be useful to develop a robust library specific to *D. magna* to improve our (and future) results. As a future perspective, proteomics studies should be used in order to determine mechanisms of action of carbendazim on *D. magna.*

Acknowledgments

This work was supported by the project RePulse- Responses of *Daphnia magna* exposed to chemical pulses and mixtures throughout generations (FCOMP-01-0124-FEDER-019321; Refª. FCT PTDC/AAC-AMB/117178/2010), by funding FEDER through COMPETE- Programa Operacional Factores de Competitividade, and by the Portuguese Science Foundation (FCT) through CESAM: UID/AMB/50017/2013. Ana Rita Rego Gouveia Silva was funded by the project "MARPRO- Conservation of marine protected species in Mainland Portugal" through the doctoral fellowship (BD/UI88/5534/2011). Ana Raquel Soares was funded by an individual post-doctoral grant SFRH/BPD/77528/2011. Patrícia Silva was funded by an individual doctoral grant SFRH/BD/52571/2014.

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Supplementary data

Table 6.1SD: Full list of differentially expressed genes following exposure of *Daphnia magna* to carbendazim (CBZ) **a)** F0 generation (191 up-regulated and 98 down-regulated genes) **b)** F12 generation (53 up-regulated and 66 down-regulated genes). Up-regulation was considered whenever the fold change was higher than 1.5, whereas down-regulation was considered whenever the fold change was below -1.5. The arrows refer to up- (↑) or down-regulated (↓) genes compared to clean medium.

a) F0 generation

Gene ID	Gene description [species]	CBZ (Fold change)
T32007	hypothetical protein F36H9.6 [*Caenorhabditis elegans*]	↑ 18.95
XP_973543	polypeptide N-acetylgalactosaminyltransferase 5 [*Tribolium castaneum*]	↑ 18.77
YP_548045	hypothetical protein Bpro_1196, membrane protein [*Polaromonas* sp.]	↑ 18.04
XP_384927	hypothetical protein [*Fusarium graminearum*]	↑ 15.59
XP_966633	histone deacetylase Rpd3 [*Tribolium castaneum* (red flour beetle)]	↑ 14.83
XP_785816	PREDICTED: similar to muscle Y-box protein YB2 [*Strongylocentrotus purpuratus*]	↑ 14.83
YP_052887	rps7 ribosomal protein S7 [*Saprolegnia ferax*]	↑ 14.18
NP_001018342	tas2r203 taste receptor, type 2, member 203 [*Danio rerio* (zebrafish)]	↑ 14.01
XP_393137	UPF0183 protein CG7083-like [*Apis mellifera* (honey bee)]	↑ 13.92
ZP_00592874	GCN5-related N-acetyltransferase [*Prosthecochloris aestuarii*]	↑ 13.72
AAH76191	thoc7 THO complex 7 [*Danio rerio* (zebrafish)]	↑ 13.27
XP_755614	AFUA_2G12830 UDP-glucosyl transferase family protein [*Aspergillus fumigatus*]	↑ 13.07
YP_660772	hypothetical protein [*Pseudoalteromonas atlantica*]	↑ 12.85
XP_392758	S-phase kinase-associated protein 1 [*Apis mellifera* (honey bee)]	↑ 12.75
EAT38914	AAEL009235-PA [*Aedes aegypti* (yellow fever mosquito)]	↑ 12.49
AAV34845	RpL34 ribosomal protein L34 [*Bombyx mori* (domestic silkworm)]	↑ 12.20
CAG10013	unnamed protein product [*Tetraodon nigroviridis*]	↑ 12.18
CAG01937	unnamed protein product [*Tetraodon nigroviridis*]	↑ 12.18
XP_397060	uncharacterized [*Apis mellifera* (honey bee)]	↑ 12.04
AAH62838	hsdl2 hydroxysteroid dehydrogenase like 2 [*Danio rerio* (zebrafish)]	↑ 12.00
BAE38837	Zranb2 zinc finger, RAN-binding domain containing 2 [*Mus musculus* (house mouse)]	↑ 11.90
AAX28551	SJCHGC05463 protein [*Schistosoma japonicum*]	↑ 11.84
XP_623241	growth hormone-inducible transmembrane protein-like [*Apis mellifera* (honey bee)]	↑ 11.75
BAB21109	Ef-1d elongation factor 1 delta [*Bombyx mori* (domestic silkworm)]	↑ 11.59
AAC28351	cytochrome P450 [*Homarus americanus*]	↑ 11.59
XP_455853	hypothetical protein [*Kluyveromyces lactis*]	↑ 11.44
XP_970811	vesicle transport protein SEC20 [*Tribolium castaneum* (red flour beetle)]	↑ 11.42
ZP_01120123	hypothetical protein RB2501_07115 [*Robiginitalea biformata*]	↑ 11.15
AAH41737	eif5 eukaryotic translation initiation factor 5 [*Xenopus laevis* (African clawed frog)]	↑ 10.81
XP_363794	hypothetical protein MG01720.4 [*Magnaporthe grisea*]	↑ 10.58
XP_971073	AP-1 complex subunit sigma-2 [*Tribolium castaneum* (red flour beetle)]	↑ 10.55
AAQ22478	PlexB Plexin B [*Drosophila melanogaster* (fruit fly)]	↑ 10.53
XP_623750	myotrophin-like [*Apis mellifera* (honey bee)]	↑ 10.48
EAA07972	MMSA_ANOGA AGAP002499-PA [*Anopheles gambiae str. PEST*]	↑ 10.37
NP_503838	G-protein coupled receptor. Protein C50H11.13. species: *Caenorhabditis elegans*	↑ 10.36
AAN37244	PF14_0631 conserved Plasmodium protein, unknown function [*Plasmodium falciparum*]	↑ 10.33
ABF51517	legumaturain [*Bombyx mori* (domestic silkworm)]	↑ 10.31
XP_397115	PREDICTED: similar to ENSANGP00000014264 [*Apis mellifera*]	↑ 10.29
ZP_01117305	Zebrafish DNA sequence from clone CH211-69O18 in linkage group 16, complete sequence	↑ 10.21
EAL26005	Dpse\GA14100 [*Drosophila pseudoobscura pseudoobscura*]	↑ 10.13
EAS03921	TTHERM_00455600 HMG box protein [*Tetrahymena thermophila*]	↑ 10.12
EAT48786	AaeL_AAEL000159 AAEL000159-PA [*Aedes aegypti* (yellow fever mosquito)]	↑ 10.04

AAI16802	Mageb18 melanoma antigen family B, 18 [*Mus musculus* (house mouse)]	↑ 9.90
EAA10370	ENSANGP00000025920 [*Anopheles gambiae* str. PEST]	↑ 9.89
XP_624856	BTB/POZ domain-containing protein KCTD5-like [*Apis mellifera* (honey bee)]	↑ 9.87
CAH90002	EIF4A2 eukaryotic translation initiation factor 4A2 [*Pongo abelii* (Sumatran orangutan)]	↑ 9.85
ZP_01233257	hypothetical protein VAS14_10384 [Vibrio angustum]	↑ 9.80
EAA05974	40S ribosomal protein S3a [*Tribolium castaneum* (red flour beetle)]	↑ 9.74
AAY63979	glycerol-3-phosphate dehydrogenase [*Lysiphlebus testaceipes*]	↑ 9.74
XP_479530	putative potassium transporter [*Oryza sativa* (japonica cultivar-group)]	↑ 9.56
AAS91007	slowmo [*Bombyx mori* (domestic silkworm)]	↑ 9.41
XP_793079	transcription factor AP-1-like [*Strongylocentrotus purpuratus*(purple sea urchin)]	↑ 9.40
XP_968064	40S ribosomal protein S3a [*Tribolium castaneum* (red flour beetle)]	↑ 9.34
NP_652184	oxidase. Probable cytochrome c oxidase subunit 7A, mitochondrial [*Drosophila melanogaster*]	↑ 9.31
XP_850642	PREDICTED: similar to GDP-mannose pyrophosphorylase B isoform 2 isoform 2 [*Canis familiaris*]	↑ 9.28
P25169	Sodium/potassium-transporting ATPase subunit beta (Sodium/potassium-dependent ATPase beta subunit) [*Artemia* sp.] Na+/K+-exchanging ATPase (EC 3.6.3.9) beta chain - brine shrimp	↑ 9.28
XP_397220	Surf1 surfeit 1 [*Apis mellifera* (honey bee)]	↑ 9.27
XP_700569	PREDICTED: similar to alpha-2-macroglobulin receptor [*Danio rerio* (zebrafish)]	↑ 9.27
CAD70781	hypothetical protein [*Neurospora crassa*]	↑ 9.11
XP_953782	TA16735 hypothetical protein [*Theileria annulata strain Ankara*]	↑ 9.06
EAT43245	stretch regulated skeletal muscle protein, putative [*Aedes aegypti*]	↑ 9.02
XP_797717	Golgi SNAP receptor complex member 1 [*Strongylocentrotus purpuratus* (purple sea urchin)]	↑ 8.93
XP_604956	NPAT nuclear protein, ataxia-telangiectasia locus [*Bos taurus* (cattle)]	↑ 8.89
XP_682935	solute carrier family 35 member F1 [*Danio rerio*]	↑ 8.86
ZP_01065094	hypothetical protein MED222_15549 [*Vibrio* sp.]	↑ 8.79
XP_751302	AFUA_6G14280 flavin-binding monooxygenase-like protein [*Aspergillus fumigatus*]	↑ 8.75
EAA08286	ENSANGP00000017110 [*Anopheles gambiae* str]	↑ 8.62
BAD18123	DESAT4 fatty acid desaturase [*Bombyx mori* (domestic silkworm)]	↑ 8.57
EAR91724	TTHERM_00396960 kinase domain protein [*Tetrahymena thermophila*]	↑ 8.54
CAF94261	unnamed protein product [*Tetraodon nigroviridis*]	↑ 8.35
AAC79426	phosphate transport protein [*Choristoneura fumiferana*]	↑ 8.30
ZP_01223527	4-carboxy-2-hydroxymuconate-6-semialdehyde dehydrogenase [marine gamma proteobacterium]	↑ 8.28
CAE67987	Hypothetical protein CBG13597 [*Caenorhabditis briggsae*]	↑ 8.15
AAN79130	Bacteriophage N4 adsorption protein B [*Escherichia coli*]	↑ 8.06
EAA07158	AgaP_AGAP010476 AGAP010476-PA [*Anopheles gambiae str. PEST*]	↑ 8.05
XP_624608	renin receptor-like [*Apis mellifera* (honey bee)]	↑ 8.00
AAK27862	Hypothetical protein Y37E3.4 [*Caenorhabditis elegans*]	↑ 8.00
XP_791551	cytochrome c1, heme protein, mitochondrial [*Strongylocentrotus purpuratus* (purple sea urchin)]	↑ 7.97
XP_973533	malate dehydrogenase, mitochondrial [*Tribolium castaneum* (red flour beetle)]	↑ 7.80
ZP_00510523	Cell division FtsK/SpoIIIE protein [*Clostridium thermocellum*]	↑ 7.60
BAD63461	phage-related protein [*Bacillus clausii*]	↑ 7.59
XP_967013	PREDICTED: similar to CG9160-PA, isoform A [*Tribolium castaneum*]	↑ 7.59
XP_966534	ethanolamine-phosphate cytidylyltransferase [*Tribolium castaneum* (red flour beetle)]	↑ 7.58
CAF97221	unnamed protein product [*Tetraodon nigroviridis*]	↑ 7.55
AAX57282	CT099 [*Lycopersicon peruvianum*]	↑ 7.53
XP_974308	metaxin-2-like [*Tribolium castaneum* (red flour beetle)]	↑ 7.48
NP_077791	Lipoyl synthase, mitochondrial [*Mus musculus*]	↑ 7.47
XP_995118	PREDICTED: similar to CG13957-PA [*Mus musculus*]	↑ 7.45
XP_392882	calcyphosin-like protein-like [*Apis mellifera* (honey bee)]	↑ 7.38
XP_967422	DNA replication licensing factor Mcm7 [*Tribolium castaneum* (red flour beetle)]	↑ 7.34
AAY60144	oocyte maturation factor Mos [*Anas poecilorhyncha*]	↑ 7.30
ZP_00134041	COG1444: Predicted P-loop ATPase fused to an acetyltransferase [*Actinobacillus pleuropneumoniae*]	↑ 7.26
XP_679830	hypothetical protein [*Plasmodium berghei ANKA*]	↑ 7.25
NP_723776	Vha68-2 CG3762-PC, isoform C; ATP synthase, anion channel, ligand-gated ion channel, DNA binding protein, hydrolase. Subfamily: V-type proton atpase catalytic subunit A (PTHR15184:SF7). [*Drosophila melanogaster*]	↑ 7.19

EAS00570	cyclic nucleotide-binding domain protein [*Tetrahymena thermophila*]	↑ 7.09
XP_624674	translocon-associated protein subunit gamma-like [*Apis mellifera* (honey bee)]	↑ 7.04
XP_393411	ubiquitin-conjugating enzyme E2 variant 2-like [*Apis mellifera* (honey bee)]	↑ 6.98
XP_623978	PREDICTED: similar to ENSANGP00000011134 [*Apis mellifera* (honey bee)]	↑ 6.93
AAI14116	Solute carrier family 25 (mitochondrial thiamine pyrophosphate carrier), member 19 [*Bos taurus* (cattle)]	↑ 6.87
AAO27090	CTP synthase [*Buchnera aphidicola* str]	↑ 6.82
XP_001072503	PREDICTED: similar to putative MAPK activating protein PM20,PM21 isoform 1 [*Rattus norvegicus*]	↑ 6.79
EAA12371	AGAP008234-PA [*Anopheles gambiae str. PEST*]	↑ 6.60
ABF51368	H^+ transporting ATP synthase O subunit [*Bombyx mori* (domestic silkworm)]	↑ 6.37
XP_973734	myosin-2 essential light chain [*Tribolium castaneum* (red flour beetle)]	↑ 6.34
XP_681269	hypothetical protein [*Aspergillus nidulans*]	↑ 6.32
XP_790964	copine-8 [*Strongylocentrotus purpuratus* (purple sea urchin)]	↑ 6.26
BAC60471	fadJ multifunctional fatty acid oxidation complex subunit alpha [*Vibrio parahaemolyticus*]	↑ 6.24
ZP_01304133	xylosidase/arabinosidase [*Sphingomonas* sp.]	↑ 6.21
XP_672403	PB300124.00.0 hypothetical protein [*Plasmodium berghei ANKA*]	↑ 6.20
XP_392015	chloride channel protein 2 [*Apis mellifera* (honey bee)]	↑ 6.17
YP_581567	Pcryo_2306 putative DNA helicase [*Psychrobacter cryohalolentis*]	↑ 6.09
XP_725993	PY05524 hypothetical protein [*Plasmodium yoelii yoelii 17XNL*]	↑ 6.05
XP_969486	PREDICTED: similar to Jagged-1 precursor (Jagged1) [Tribolium castaneum]	↑ 6.00
EAT43058	AAEL005474-PA [*Aedes aegypti* (yellow fever mosquito)]	↑ 5.98
BAE02066	uncharacterized [*Macaca fascicularis* (crab-eating macaque)]	↑ 5.87
BAB77866	alr1500 hypothetical protein [*Nostoc* sp.]	↑ 5.85
ABB06938	FAD dependent oxidoreductase [*Burkholderia lata*]	↑ 5.83
CAG09120	unnamed protein product [*Tetraodon nigroviridis*]	↑ 5.80
ABC73068	venom allergen 5 [*Vespula maculifrons*]	↑ 5.78
ABD19264	cytochrome b [*Daphnia pulex*]	↑ 5.76
AAB31526	O-type P element protein {exons 0-3} [*Drosophila bifasciata*]	↑ 5.76
AAC05908	cytochrome oxidase subunit II [*Pieris rapae*]	↑ 5.71
CAB05290	hypothetical protein T27E7.3 T27E7.3 [*Caenorhabditis elegans*]	↑ 5.53
BAD40302	2-oxoacid:ferredoxin oxidoreductase alpha subunit [*Symbiobacterium thermophilum*]	↑ 5.50
CAG07432	unnamed protein product [*Tetraodon nigroviridis*]	↑ 5.49
AAS93718	CG30022 [*Drosophila melanogaster* (fruit fly)]	↑ 5.46
AAH85561	Hypothetical protein [*Danio rerio* (zebrafish)]	↑ 5.41
ZP_01034049	His/Glu/Gln/Arg/opine family ABC transporter, permease protein [*Roseovarius* sp.]	↑ 5.39
EAA08205	AGAP002490-PA [*Anopheles gambiae str. PEST*]	↑ 5.37
XP_394362	E3 ubiquitin-protein ligase UBR1 [*Apis mellifera* (honey bee)]	↑ 5.36
CAB05757	SRX-29 [*Caenorhabditis elegans*]	↑ 5.36
CAA10769	hypothetical protein [*Cryptosporidium parvum*]	↑ 5.34
AAS53791	AFR420Wp [*Ashbya gossypii* ATCC 10895] AFR420Wp [*Eremothecium gossypii*]	↑ 5.11
XP_392616	PREDICTED: similar to ENSANGP00000021560 [*Apis mellifera*]	↑ 5.11
XP_723863	PY00386 CCAAT-box DNA binding protein subunit B [*Plasmodium yoelii yoelii*]	↑ 5.11
XP_636901	DDB_G0288093 RING zinc finger-containing protein [*Dictyostelium discoideum*]	↑ 5.07
CAH03604	PTMB.407 hypothetical protein [*Paramecium tetraurelia strain d4-2*]	↑ 5.04
NP_440321	Sll1510 protein [*Synechocystis*]	↑ 5.03
XP_624692	Glutathione S-transferase T1 [*Apis mellifera* (honey bee)]	↑ 5.02
EAS03884	TTHERM_00455230 hypothetical protein [*Tetrahymena thermophila*]	↑ 5.00
Q25158	Compound eye opsin BCRH2 opsin BcRh2 [*Hemigrapsus sanguineus*]	↑ 4.87
XP_394551	NEDD8-conjugating enzyme UBE2F-like [*Apis mellifera* (honey bee)]	↑ 4.75
EAT45700	AAEL003027-PA [*Aedes aegypti* (yellow fever mosquito)]	↑ 4.74
XP_968298	PREDICTED: similar to CG31543-PC, isoform C [*Tribolium castaneum*]	↑ 4.71
XP_781103	homogentisate 1,2-dioxygenase [*Strongylocentrotus purpuratus* (purple sea urchin)]	↑ 4.64
EAA00702	AGAP011988-PA [*Anopheles gambiae str. PEST*]	↑ 4.57
EAR85282	TTHERM_00470550 phospholipid-translocating P-type ATPase, flippase family protein [*Tetrahymena thermophila*]	↑ 4.57
YP_547797	Bpro_0943 hypothetical protein [*Polaromonas* sp.]	↑ 4.56
AAR33556	cytochrome c oxidase, coo3-type, cytochrome c subunit II, one heme-binding site [*Geobacter*	↑ 4.55

	sulfurreducens PCA]	
NP_194153	3-oxo-Delta(4,5)-steroid 5-beta-reductase. [*Arabidopsis thaliana*]	↑ 4.53
ABA45367	zinc ABC transporter ATP-binding protein [*Streptococcus agalactiae*]	↑ 4.47
ZP_01189085	Excinuclease ABC, A subunit [*Halothermothrix orenii*]	↑ 4.44
EAT43025	AaeL_AAEL005513 AAEL005513-PA [*Aedes aegypti* (yellow fever mosquito)]	↑ 4.44
XP_710938	Potential fungal zinc cluster transcription factor [*Candida albicans*]	↑ 4.40
XP_751922	aminopeptidase [*Aspergillus fumigatus*]	↑ 4.39
AAT74669	cysteine-rich secreted protein 3 [*Mesocestoides vogae*]	↑ 4.39
AAH19729	Usf2 upstream transcription factor 2 [*Mus musculus* (house mouse)]	↑ 4.29
NP_648180	CG13675-PA [*Drosophila melanogaster*]	↑ 4.03
XP_958062	NCU10014 hypothetical protein [*Neurospora crassa*]	↑ 3.92
EAA00530	AGAP012418-PA [*Anopheles gambiae str. PEST*]	↑ 3.89
AAY54998	IP06749p [*Drosophila melanogaster*]	↑ 3.87
BAC24521	minC [*Wigglesworthia glossinidia* endosymbiont of *Glossina brevipalpis*]	↑ 3.86
XP_001069615	PREDICTED: similar to YY1 transcription factor [*Rattus norvegicus*]	↑ 3.86
BAC12610	hypothetical conserved protein [*Oceanobacillus iheyensis* HTE831]	↑ 3.69
YP_629939	MXAN_1687 hypothetical protein [*Myxococcus xanthus* DK 1622]	↑ 3.53
AAZ06578	pAW63_007 type II intron reverse transcriptase maturase [*Bacillus thuringiensis* serovar kurstaki]	↑ 3.52
YP_621339	Bcen_1460 MscS mechanosensitive ion channel [*Burkholderia cenocepacia*]	↑ 3.48
XP_392121	papilin-like [*Apis mellifera* (honey bee)]	↑ 3.41
AAT89163	hypothetical protein [*Leifsonia xyli* subsp.]	↑ 3.39
XP_763925	TP04_0290 hypothetical protein [*Theileria parva strain Muguga*]	↑ 3.36
AAY66970	secreted protein [*Ixodes scapularis*]	↑ 3.30
XP_695583	similar to HYLS1 protein [*Danio rerio* (zebrafish)]	↑ 3.28
EAA04645	AGAP007365-PA [*Anopheles gambiae str. PEST*]	↑ 3.24
XP_785823	PREDICTED: similar to dispatched homolog 1 [*Strongylocentrotus purpuratus*]	↑ 3.19
XP_521564	Centrosomal protein 55kDa [*Pan troglodytes]*	↑ 3.14
XP_396152	SMSr sphingomyelin synthase-related 1 [*Apis mellifera* (honey bee)]	↑ 3.06
NP_872374	hypothetical protein [Homo sapiens]	↑ 3.03
AAH82673	bcat1 branched chain amino-acid transaminase 1, cytosolic [*Xenopus laevis* (African clawed frog)]	↑ 2.90
BAD46348	Os09g0525400 [*Oryza sativa Japonica Group*]	↑ 2.87
CAG67999	conserved hypothetical protein; putative membrane protein [*Acinetobacter* sp. ADP1]	↑ 2.83
EAL33401	GA18290-PA [Drosophila pseudoobscura]	↑ 2.82
EAT38039	AaeL_AAEL010027 AAEL010027-PA [*Aedes aegypti* (yellow fever mosquito)]	↑ 2.79
YP_293946	EhV192 hypothetical protein [*Emiliania huxleyi virus 86*]	↑ 2.77
EAA04917	AgaP_AGAP000973 AGAP000973-PA [*Anopheles gambiae str. PEST*]	↑ 2.75
EAL27357	gene product from transcript GA20418-RA [*Drosophila pseudoobscura*]	↑ 2.47
ZP_01079163	possible helicase [*Synechococcus* sp.]	↑ 2.47
EAT42364	AAEL006097-PA [*Aedes aegypti* (yellow fever mosquito)]	↑ 2.21
AAT39336	DNA repair protein RAD51 [*Oikopleura dioica*]	↑ 2.20
CAA93496	ALG-1 [*Caenorhabditis elegans*]	↑ 2.17
XP_952900	TA07340 hypothetical protein [*Theileria annulata strain Ankara*]	↑ 2.01
EAR90129	TTHERM_00354760 hypothetical protein [*Tetrahymena thermophila*]	↑ 1.89

Gene ID	**Gene description [species]**	**CBZ**
NP_610462	shrb transfer/carrier protein [*Drosophila melanogaster*]	↓ -2.03
EAS00551	hypothetical protein TTHERM_00409040 [*Tetrahymena thermophila*]	↓ -2.03
AAH42230	Ribosomal protein S1a protein [*Xenopus laevis*]	↓ -2.10
XP_458550	hypothetical protein DEHA0D02585g [*Debaryomyces hansenii*]	↓ -2.14
XP_821615	protein kinase [*Trypanosoma cruzi* strain CL Brener]	↓ -2.14
XP_592181	PREDICTED: similar to Y37D8A.2 isoform 1 [*Bos taurus*]	↓ -2.17
XP_686827	PREDICTED: similar to Bmp1 protein [*Danio rerio*]	↓ -2.20
XP_966285	DNA polymerase epsilon catalytic subunit A [*Plasmodium falciparum*]	↓ -2.21
XP_678020	mitochondrial DNA, complete genome [*Triops cancriformis*]	↓ -2.21
XP_541754	guanyl-nucleotide exchange factor [*Canis familiaris*]	↓ -2.27
XP_651053	hypothetical protein 185.t00007 [*Entamoeba histolytica*]	↓ -2.27
NP_079004	succinyl-CoA:glutarate-CoA transferase [*Homo sapiens*]	↓ -2.30

AAH97162	F-box and leucine-rich repeat protein 18 [*Danio rerio*]	↓ -2.33
ZP_00131989	Predicted ATPase [*Haemophilus somnus*]	↓ -2.40
XP_974187	PREDICTED: similar to CG8029-PB, isoform B [*Tribolium castaneum*]	↓ -2.42
NP_766109	nuclear protein in testis [*Mus musculus*]	↓ -2.43
XP_640534	GMP synthetase [*Dictyostelium discoideum*]	↓ -2.44
AAM40505	DNA mismatch repair protein [*Xanthomonas campestris*]	↓ -2.63
BAC10625	ubiquitin conjugating enzyme-like protein [*Bombyx mori*]	↓ -2.64
BAC98829	F-box WD40 protein [*Labidochromis caeruleus*]	↓ -2.65
XP_623472	PREDICTED: similar to ENSANGP00000024947 [*Apis mellifera*]	↓ -2.65
CAI86921	conserved protein of unknown function ; putative membrane protein [*Pseudoalteromonas haloplanktis*]	↓ -2.69
CAE79263	glutaminyl-tRNA synthetase [*Bdellovibrio bacteriovorus*]	↓ -2.71
NP_701577	hypothetical protein PFL1075w [*Plasmodium falciparum*]	↓ -2.71
EAL28674	GA15521-PA [*Drosophila pseudoobscura*]	↓ -2.72
CAE66221	Hypothetical protein CBG11463 [*Caenorhabditis briggsae*]	↓ -2.72
NP_001017150	chromobox homolog 1 [*Xenopus tropicalis*]	↓ -2.77
XP_393841	PREDICTED: similar to AMME syndrome candidate gene 1 protein [*Apis mellifera*]	↓ -2.81
AAT39415	Gaba(A) receptor associated protein [*Branchiostoma belcheri tsingtaunese*]	↓ -2.83
EAT36570	alcohol dehydrogenase [*Aedes aegypti*]	↓ -2.86
XP_624527	repressor of RNA polymerase III transcription MAF1 homolog [*Apis mellifera* (honey bee)]	↓ -2.87
AAD32568	NT6 [Nicotiana tabacum]	↓ -2.88
XP_859489	PREDICTED: similar to hydroxyacyl dehydrogenase, subunit A isoform 3 [*Canis familiaris*]	↓ -2.91
XP_624997	PREDICTED: similar to oxysterol-binding protein-like protein 9 isoform d [*Apis mellifera*]	↓ -3.00
EAR96527	Major Facilitator Superfamily protein [*Tetrahymena thermophila*]	↓ -3.02
XP_967132	PREDICTED: similar to CG6016-PB, isoform B isoform 1 [*Tribolium castaneum*]	↓ -3.03
NP_851603	microtubule associated serine/threonine kinase 1 [*Rattus norvegicus*]	↓ -3.03
AAK41879	Conserved hypothetical protein [*Sulfolobus solfataricus*]	↓ -3.05
NP_523530	60S ribosomal protein L13 RPL13 [*Drosophila melanogaster*]	↓ -3.12
XP_974675	zinc transporter ZIP1 [*Tribolium castaneum*]	↓ -3.15
NP_701446	hypothetical protein PFL0405w [*Plasmodium falciparum*]	↓ -3.20
XP_361380	hypothetical protein MG03854.4 [*Magnaporthe grisea*]	↓ -3.26
AAH50515	WD repeat domain 8 [*Danio rerio*]	↓ -3.34
XP_362894	hypothetical protein MG08543.4 [*Magnaporthe grisea*]	↓ -3.39
CAA67766	acute phase serum amyloid A (SAA) [*Oncorhynchus mykiss*]	↓ -3.39
AAH77956	MGC80949 protein [*Xenopus laevis*]	↓ -3.41
XP_971851	PREDICTED: similar to NADH-ubiquinone oxidoreductase 42 kDa subunit, mitochondrial precursor (Complex I-42KD) (CI-42KD) [*Tribolium castaneum*]	↓ -3.42
EAT34816	glycine cleavage system h protein [*Aedes aegypti*]	↓ -3.45
CAG31427	annexin A11 [*Gallus gallus*]	↓ -3.46
XP_650833	heat shock protein 70 [*Entamoeba histolytica*]	↓ -3.47
YP_485288	pyrroloquinoline-quinone aldehyde dehydrogenase [*Rhodopseudomonas palustris*]	↓ -3.49
AAV34884	ribosomal protein S27 [*Bombyx mori*]	↓ -3.49
XP_969209	PREDICTED: similar to sphingosine-1-phosphatase [*Tribolium castaneum*]	↓ -3.50
XP_975592	PREDICTED: similar to CG40410-PA.3 [*Tribolium castaneum*]	↓-3.51
XP_221438	PREDICTED: similar to Cdc42 GTPase-activating protein [*Rattus norvegicus*]	↓ -3.51
CAC44629	deafness dystonia protein [*Takifugu rubripes*]	↓ -3.68
XP_974201	39S ribosomal protein L44, mitochondrial [*Tribolium castaneum*]	↓-3.68
EAA13751	AGAP010769-PA glucosamine 6-phosphate N-acetyltransferase [*Anopheles gambiae* str. PEST]	↓ -3.75
EAT40746	conserved hypothetical protein [*Aedes aegypti*]	↓ -3.78
XP_975769	cytochrome b-c1 complex subunit 2, mitochondrial [Tribolium castaneum]	↓ -3.79
XP_001076360	PREDICTED: similar to retinoblastoma binding protein 6 isoform 1 isoform 2 [*Rattus norvegicus*]	↓ -3.97
XP_653493	hypothetical protein 81.t00020 [*Entamoeba histolytica*]	↓ -3.98
XP_971017	zinc transporter ZIP11 [*Tribolium castaneum*]	↓ -4.00
EAL27218	GA18926-PA [*Drosophila pseudoobscura*]	↓ -4.12
ZP_00800575	4Fe-4S ferredoxin, iron-sulfur binding [*Alkaliphilus metalliredigenes*]	↓ -4.16
XP_829758	hypothetical protein Tb11.01.8780 [*Trypanosoma brucei*]	↓ -4.22
XP_726251	hypothetical protein PY00679 [*Plasmodium yoelii yoelii* str.]	↓-4.24
BAD94515	peroxisome proliferator-activated receptor gamma [*Oncorhynchus keta*]	↓ -4.33
CAD67790	No homology	↓ -4.39
BAB05837	hypothetical protein BH2118 [*Bacillus halodurans*]	↓ -4.41
AAT42372	glycogen synthase kinase-3 [*Lytechinus variegatus*]	↓-4.74

XP_001102209	PREDICTED: similar to Methylmalonyl-CoA epimerase, mitochondrial precursor (DL-methylmalonyl-CoA racemase) isoform 1 [*Macaca mulatta*]	↓-4.75
AAC27659	tryptophan oxygenase [*Anopheles gambiae*]	↓ -4.85
NP_039078	ORF FPV115 Ankyrin repeat gene family protein [*Fowlpox* virus]	↓ -4.90
XP_667711	hypothetical protein Chro.70604 [*Cryptosporidium hominis*]	↓ -4.96
ZP_01066446	putative permease [*Vibrio* sp.]	↓ -5.13
XP_696756	PREDICTED: similar to sulfiredoxin 1 homolog [*Danio rerio*]	↓ -5.26
ABD33303	hypothetical protein MtrDRAFT_AC158502g12v1 [*Medicago truncatula*]	↓ -5.32
XP_388815	hypothetical protein FG08639.1 [*Gibberella zeae* PH-1]	↓ -5.47
CAE73165	Hypothetical protein CBG20561 [*Caenorhabditis briggsae*]	↓ -7.21
XP_851407	PREDICTED: similar to serine/cysteine proteinase inhibitor, clade I, member 2 isoform 1 [*Canis familiaris*]	↓ -9.10
AAB01338	EGF repeat transmembrane protein [*Mus musculus*]	↓ -9.69
AAH81106	MGC83377 protein [*Xenopus laevis*]; solute carrier family 5 (sodium/glucose cotransporter), member 2	↓ -12.11
EAT39824	inorganic-stress: Fullerene nanoparticle, normal: Females-adult [*Aedes aegypti*]	↓ -17.08
XP_420864	T-cell surface glycoprotein CD8 alpha chain-like; PREDICTED: similar to CD8 alpha chain precursor [*Gallus gallus*]	↓ -24.06
AAP77784	hypothetical protein HH_1187 [*Helicobacter hepaticus*]	↓ -7396.75
XP_700169	PREDICTED: similar to conserved hypothetical protein [*Danio rerio*]	↓ -10187.81
AAR01249	laccase 8 [*Coprinopsis cinerea*]	↓ -10930.12
AAH00967	NudC domain containing 1 [*Homo sapiens*]	↓ -11402.40
XP_500810	SEC16, ISOFORM F; hypothetical protein [*Yarrowia lipolytica*]	↓ -13979.40
AAQ75727	NADH dehydrogenase I [*Errhomus variabilis*]	↓ -20664.18
XP_784306	PREDICTED: similar to placental protein 11 related [*Strongylocentrotus purpuratus*]	↓ -29883.84
ZP_01181739	Phage minor structural protein, N-terminal [*Bacillus cereus* subsp.]	↓ -35745.20
XP_765680	hypothetical protein TP01_0153 [*Theileria parva* strain Muguga]	↓ -59189.85
AAM51523	Hypothetical protein C28G1.6 [*Caenorhabditis elegans*]	↓ -69117.57
ZP_00851049	hypothetical protein Shewana3DRAFT_1897 [*Shewanella* sp.]	↓ -98465.68
XP_385040	hypothetical protein FG04864.1 [*Gibberella zeae*]	↓ -109521.08
XP_973845	apoptosis-resistant E3 ubiquitin protein ligase 1 [*Tribolium castaneum*]	↓ -116578.52

b) F12 generation

Gene ID	Gene description [species]	CBZ (fold change)
EAA04403	AGAP006931-PA; ENSANGP00000021782 [*Anopheles gambiae* str. PEST]	↑ 72368.51
YP_548045	hypothetical protein Bpro_1196 [*Polaromonas* sp.]	↑ 71962.22
EAL26781	GA20714-PA [*Drosophila pseudoobscura*]	↑ 56738.63
CAD67790	double stranded RNA-activated protein kinase 1 [*Tetraodon nigroviridis*]	↑ 35149.50
XP_827078	hypothetical protein Tb09.160.5290 [*Trypanosoma brucei*]	↑ 28898.11
XP_665805	hypothetical protein Chro.60399 [*Cryptosporidium hominis*]	↑ 22473.06
AAM74161	Pax-6 protein [*Euprymna scolopes*]	↑ 18679.08
YP_476405	ABC1 domain protein [*Synechococcus* sp.]	↑ 18288.22
ABG52453	peptidase M23B [*Trichodesmium erythraeum*]	↑ 17199.64
BAE56654	unnamed protein product [Aspergillus oryzae]	↑ 17137.55
CAG76660	conserved hypothetical protein [*Erwinia carotovora* subsp. atroseptica]	↑ 16193.51
NP_008814	NADH dehydrogenase subunit 6 [*Mustelus manazo*]	↑ 15117.52
XP_394766	PREDICTED: similar to CG33175-PG, isoform G, partial [*Apis mellifera*]	↑ 14893.60
AAH88813	lectin, galactoside-binding, soluble, 9B;; Lgals9-prov protein [*Xenopus tropicalis*]	↑ 14143.80
EAR91277	transmembrane protein, putative; hypothetical protein TTHERM_00784640 [*Tetrahymena thermophila*]	↑10882.44
NP_922637	hypothetical protein [*Oryza sativa* (japonica cultivar-group)]	↑ 10539.98
XP_765680	hypothetical protein TP01_0153 [*Theileria parva* strain Muguga]	↑ 10199.45
NP_492661	MUTator family member (mut-16) [*Caenorhabditis elegans*]	↑ 8548.91
AAT64428	pMGA 1.4 [*Mycoplasma gallisepticum*] haemagglutinin homologue	↑ 8363.90
CAD51508	asparagine--tRNA ligase, putative [*Plasmodium falciparum*]	↑ 7605.56
ZP_00637578	hypothetical protein SfriDRAFT_3544 [*Shewanella frigidimarina*]	↑ 5909.83
ABB44488	Suden_1210 hypothetical protein [*Sulfurimonas denitrificans*]	↑ 4916.25
BAD94515	peroxisome proliferator-activated receptor gamma [*Oncorhynchus keta*]	↑ 4835.57
AAY54998	IP06749p [*Drosophila melanogaster*]	↑ 3815.65

AAH74846	transmembrane protease, serine 3; Transmembrane protease, serine 3, isoform 1 [*Homo sapiens*]	↑ 3770.56
XP_465194	putative speckle-type POZ protein [*Oryza sativa* (japonica cultivar-group)]	↑ 3072.81
P05842	Putative noncapsid protein NS-1 (Nonstructural protein NS1) (NCVP1)	↑ 2264.67
XP_001091323	solute carrier family 15, member 5 [*Macaca mulatta*]	↑ 10.76
XP_785823	PREDICTED: similar to dispatched homolog 1 [*Strongylocentrotus purpuratus*]	↑ 9.43
BAD74252	type IIs restriction endonuclease; type IIs restriction endonuclease [*Geobacillus kaustophilus*]	↑ 7.78
XP_430000	PREDICTED: hypothetical protein [*Gallus gallus*]	↑ 7.35
XP_981685	PREDICTED: hypothetical protein [*Mus musculus*]	↑ 6.23
XP_001069615	PREDICTED: similar to YY1 transcription factor [*Rattus norvegicus*]	↑ 6.15
ZP_01001041	LacI family regulatory protein [*Oceanicola batsensis*]	↑ 5.16
AAY66970	secreted protein [*Ixodes scapularis*]	↑ 3.37
XP_001063788	Keratin associated protein 20-like 2 [*Rattus norvegicus* (Norway rat)]	↑ 3.29
AAH70339	Deoxyuridine triphosphatase DUT [*Homo sapiens*]	↑ 3.21
CAC95124	TIR/NBS/LRR protein [*Populus deltoides*]	↑ 3.09
EAT41358	AAEL007022-PA [*Aedes aegypti*(yellow fever mosquito)]	↑ 2.80
AAK52091	Cth cystathionine gamma-lyase [*Rattus norvegicus* (Norway rat)]	↑ 2.74
XP_746198	PC001062.02.0 hypothetical protein [*Plasmodium chabaudi chabaudii*]	↑ 2.71
T44130	hypothetical protein [imported] - [*Staphylococcus aureus*] (fragment)	↑ 2.63
XP_813347	UDP-glucoronosyl and UDP-glucosyl transferase [*Trypanosoma cruzi* strain CL Brener]	↑ 2.42
XP_541754	guanyl-nucleotide exchange factor [*Canis familiaris*]	↑ 2.40
XP_969056	ubiquitin carboxyl-terminal hydrolase 14 [*Tribolium castaneum* (red flour beetle)]	↑ 2.40
ZP_01376161	hypothetical protein Ccur5_01001129 [*Campylobacter curvus*]	↑ 2.29
XP_974201	PREDICTED: 39S ribosomal protein L44, mitochondrial [*Tribolium castaneum*]	↑ 2.25
AAZ75599	CRISP-ENH2 [*Pseudoferania polylepis*]	↑ 2.13
XP_971017	PREDICTED: zinc transporter ZIP11 isoform X2 [*Tribolium castaneum*]	↑ 2.08
AAH74592	MGC69530 protein [*Xenopus (Silurana) tropicalis*]	↑ 2.01
P34724	RecName: Full=Acid phosphatase; Flags: Precursor [*Aspergillus niger*]	↑ 2.00
XP_393267	PREDICTED: testican-1-like [*Apis mellifera*]	↑ 1.95
XP_966285	DNA polymerase epsilon catalytic subunit A [*Plasmodium falciparum*]	↑ 1.72

Gene ID	**Gene description [species]**	**CBZ**
CAE73165	Hypothetical protein CBG20561 [*Caenorhabditis briggsae*]	↓ -2.61
XP_651175	chloride channel protein 2[*Entamoeba histolytica*]	↓ -2.91
EAA08389	AGAP003192-PA [*Anopheles gambiae* str. PEST]	↓ -3.05
ABE93099	hypothetical protein MtrDRAFT_AC122172g1v2 [*Medicago truncatula*]	↓ -3.44
CAF89999	unnamed protein product [*Tetraodon nigroviridis*]	↓ -3.73
NP_440283	histidinol dehydrogenase [*Synechocystis* sp]	↓ -3.76
I30010	SMU_1069c hypothetical protein [*Streptococcus mutans* UA159]	↓ -3.77
EAT43025	mothers against dpp protein [*Aedes aegypti* (yellow fever mosquito)]	↓ -3.90
XP_581858	PREDICTED: similar to Valyl-tRNA synthetase (Valine--tRNA ligase) (ValRS) (G7a protein) [*Bos taurus*]	↓ -3.92
XP_975669	PREDICTED: hypothetical protein [*Tribolium castaneum*]	↓ -3.97
XP_973543	polypeptide N-acetylgalactosaminyltransferase 5 [*Tribolium castaneum* (red flour beetle)]	↓ -3.97
EAT34457	conserved hypothetical protein [*Aedes aegypti*]	↓ -4.01
XP_678020	hypothetical protein [*Plasmodium berghei* ANKA]	↓ -4.07
XP_710938	Potential fungal zinc cluster transcription factorspecies: Candida albicans/putative transcription factor [*Candida albicans*]	↓ -4.12
XP_640018	hypothetical protein [*Dictyostelium discoideum* AX4]	↓ -4.13
YP_547797	hypothetical protein Bpro_0943 [*Polaromonas* sp.]	↓ -4.38
ABB06938	FAD dependent oxidoreductase [*Burkholderia lata*]	↓ -4.42
NP_571006	solute carrier family 39 (zinc transporter), member 7 [*Danio rerio* (zebrafish)]	↓ -4.56
XP_956378	hypothetical protein [*Neurospora crassa* OR74A]	↓ -4.64
EAA08205	AGAP002490-PA [*Anopheles gambiae* str. PEST]	↓ -4.67
AAA29908	p48 eggshell protein	↓ -4.85
NP_650751	CG7718 [*Drosophila melanogaster*]	↓ -4.85
XP_678632	hypothetical protein [*Plasmodium berghei* ANKA]	↓ -5.05
XP_644147	vps13B hypothetical protein [*Dictyostelium discoideum*]	↓ -5.06
XP_966853	PREDICTED: similar to Longitudinals lacking protein, isoform G isoform 1 [*Tribolium castaneum*]	↓ -5.08

XP_452490	KLLA0C06567g hypothetical protein [*Kluyveromyces lactis* NRRL Y-1140]	↓ -5.36
AAL05973	peroxinectin [*Penaeus monodon*]	↓ -5.44
NP_957297	ubtfl upstream binding transcription factor, like [*Danio rerio* (zebrafish)]	↓ -5.53
XP_974970	protein bicaudal C [*Tribolium castaneum* (red flour beetle)]	↓ -5.79
XP_922294	PREDICTED: similar to pericardin CG5700-PB [*Mus musculus*]	↓ -5.83
BAB21109	Ef-1d elongation factor 1 delta [*Bombyx mori* (domestic silkworm)]	↓ -5.84
ABA25160	Transposase [*Anabaena variabilis*]	↓ -5.86
XP_995118	PREDICTED: similar to CG13957-PA [*Mus musculus*]	↓ -5.90
ZP_00052331	UDP-N-acetylglucosamine 2-epimerase [*Magnetospirillum magnetotacticum* MS-1]	↓ -5.90
AAI01850	Itgal integrin, alpha L [*Rattus norvegicus* (Norway rat)]	↓ -5.92
CAA18877	transcriptional corepressor Ssn6 [*Schizosaccharomyces pombe*]	↓ -5.97
XP_626108	hypothetical protein [*Cryptosporidium parvum* Iowa II]	↓ -6.23
ABD19264	cytochrome b [*Daphnia pulex*]	↓ -6.30
XP_972276	dolichyl pyrophosphate Man9GlcNAc2 alpha-1,3-glucosyltransferase [*Tribolium castaneum* (red flour beetle)]	↓ -6.47
CAG08737	unnamed protein product [*Tetraodon nigroviridis*]	↓ -6.58
XP_623706	crowded by cid [*Apis mellifera* (honey bee)]	↓ -6.71
CAI86226	chemotaxis protein CheY [*Pseudoalteromonas haloplanktis* TAC125]	↓ -6.89
CAK11279	novel protein similar to vertebrate topoisomerase (DNA) II beta 180kDa (TOP2B) [*Danio rerio*]	↓ -7.25
DAA04586	TPA: TPA_inf: RTN3-A2 [*Oncorhynchus mykiss*]	↓ -7.30
AAD55141	di-domain hemoglobin precursor [*Daphnia pulex*]	↓ -7.34
CAG01996	unnamed protein product [*Tetraodon nigroviridis*]	↓ -7.43
AAH85561	Hypothetical protein [*Danio rerio* (zebrafish)]	↓ -7.65
CAH07708	putative transmembrane protein [*Bacteroides fragilis*]	↓ -7.77
AAV34814	ribosomal protein L5 [*Bombyx mori* (domestic silkworm)]	↓ -7.93
ZP_01142625	hypothetical protein GuraDRAFT_1187 [*Geobacter uraniumreducens*]	↓ -8.36
XP_975561	protein fem-1 homolog B [*Tribolium castaneum* (red flour beetle)]	↓ -8.41
XP_726716	erythrocyte membrane protein [*Plasmodium yoelii yoelii*]	↓ -8.73
XP_624645	PREDICTED: similar to ribosomal protein S12 [*Apis mellifera*]	↓ -8.80
EAA13942	GPRGBB1 AGAP010281-PA [*Anopheles gambiae* str. PEST]	↓ -9.16
AAV34845	ribosomal protein L34 [*Bombyx mori*]	↓ -9.25
XP_393411	ubiquitin-conjugating enzyme E2 variant 2-like [*Apis mellifera*]	↓ -9.26
XP_395511	PREDICTED: similar to ENSANGP00000020783 [*Apis mellifera*]	↓ -9.63
XP_541851	PREDICTED: similar to Stabilin-1 precursor (FEEL-1 protein) (MS-1 antigen) [*Canis familiaris*]	↓ -9.80
XP_623241	growth hormone-inducible transmembrane protein-like; PREDICTED: similar to ENSANGP00000014774 [*Apis mellifera*]	↓ -10.04
YP_262026	2-isopropylmalate synthase [*Pseudomonas fluorescens*]	↓ -10.45
XP_975012	hydroxylysine kinase; PREDICTED: similar to CG31751-PA, isoform A [*Tribolium castaneum*]	↓ -10.47
AAV91371	hypothetical protein 3 [*Lonomia obliqua*]	↓ -11.09
XP_975116	ragulator complex protein LAMTOR5 homolog; PREDICTED: hypothetical protein [*Tribolium castaneum*]	↓ -11.74
ZP_01120123	hypothetical protein RB2501_07115 [*Robiginitalea biformata* HTCC2501]	↓ -12.23
XP_001072503	PREDICTED: similar to putative MAPK activating protein PM20,PM21 isoform 1 [*Rattus norvegicus*]	↓ -14.17
AAH29794	SH3 and cysteine rich domain 2 [*Mus musculus*]	↓ -12600.54

Table 6.2SD: Full list of differentially expressed genes. F0 *vs.* F12 generation of *Daphnia magna* are compared a) clean medium (Clean) (73 up-regulated and 62 down-regulated genes) b) carbendazim (CBZ) (119 up-regulated and 87 down-regulated genes). Up-regulation was considered whenever the fold change was higher than 1.5, whereas down-regulation was considered whenever the fold change was below -1.5. The arrows refer to up- (↑) or down-regulated (↓) genes.

a) F0 vs. F12 Clean medium

Gene ID	Gene description [species]	Clean (Fold change)
AAV34845	ribosomal protein L34 [*Bombyx mori* (domestic silkworm)]	↑ 16.29
EAA07972	MMSA_ANOGA AGAP002499-PA [*Anopheles gambiae* str. PEST]	↑ 13.23
XP_392882	calcyphosin-like protein-like [*Apis mellifera* (honey bee)]	↑ 12.78
AAF86906	triose phosphate/phosphate translocator precursor [*Mesembryanthemum crystallinum*]	↑ 12.51
AAQ22478	PlexB Plexin B [*Drosophila melanogaster* (fruit fly)]	↑ 11.79
NP_652184	oxidase. Probable cytochrome c oxidase subunit 7A, mitochondrial [*Drosophila melanogaster*]	↑ 11.62
AAV91371	hypothetical protein 3 [*Lonomia obliqua*]	↑11.45
CAE56378	Hypothetical protein CBG24057 [*Caenorhabditis briggsae*]	↑ 10.48
XP_700569	PREDICTED: similar to alpha-2-macroglobulin receptor [*Danio rerio*]	↑ 10.43
XP_393411	ubiquitin-conjugating enzyme E2 variant 2-like [*Apis mellifera* (honey bee)]	↑ 9.78
XP_797717	Golgi SNAP receptor complex member 1 [*Strongylocentrotus purpuratus* (purple sea urchin)]	↑ 9.72
AAV63979	cathepsin L1 precursor [*Artemia parthenogenetica*]	↑ 9.63
XP_605435	PREDICTED: similar to exophilin 5 [*Bos taurus*]	↑ 9.62
XP_392758	S-phase kinase-associated protein 1 [*Apis mellifera* (honey bee)]	↑ 9.54
AAC05908	cytochrome oxidase subunit II [*Pieris rapae*]	↑ 9.14
EAR94332	hypothetical protein TTHERM_00049470 [*Tetrahymena thermophila*]	↑ 9.11
EAT38914	AAEL009235-PA [*Aedes aegypti* (yellow fever mosquito)]	↑ 9.02
XP_968978	PREDICTED: similar to intracellular membrane-associated calcium-independent phospholipase A2 gamma [*Tribolium castaneum*]	↑ 9.01
XP_001072503	PREDICTED: similar to putative MAPK activating protein PM20,PM21 isoform 1 [*Rattus norvegicus*]	↑ 8.75
DAA04586	TPA: TPA_inf: RTN3-A2 [*Oncorhynchus mykiss*]	↑ 8.19
XP_757724	G-protein coupled receptor [*Ustilago maydis*]	↑ 7.92
AAI16802	melanoma antigen family B, 18 [*Mus musculus* (house mouse)]	↑ 7.89
ZP_01120123	hypothetical protein RB2501_07115 [*Robiginitalea biformata*]	↑ 7.81
NP_571006	solute carrier family 39 (zinc transporter), member 7 [*Danio rerio*]	↑ 7.81
XP_623241	growth hormone-inducible transmembrane protein-like [*Apis mellifera* (honey bee)]	↑ 7.78
BAB21109	elongation factor 1 delta [*Bombyx mori* (domestic silkworm)]	↑ 7.72
AAM44044	ferritin 2 [*Apriona germari*]	↑ 7.64
XP_967422	DNA replication licensing factor Mcm7 [*Tribolium castaneum* (red flour beetle)]	↑ 7.32
ABD19264	cytochrome b [*Daphnia pulex*]	↑ 7.32
AAL27467	TRL10 [human herpesvirus 5]	↑ 7.02
XP_534308	immunoglobulin receptor superfamily, protein phosphatase, immunoglobulin superfamily cell adhesion molecule [*Canis familiaris*]	↑ 6.98
CAG08737	unnamed protein product [*Tetraodon nigroviridis*]	↑ 6.66
XP_756287	family/subfamily: TRNA-DIHYDROURIDINE(16/17) SYNTHASE [NAD(P)(+)]-LIKE (PTHR11082:SF5) [*Ustilago maydis*]	↑ 6.64
CAF89999	unnamed protein product [*Tetraodon nigroviridis*]	↑ 6.53
AAC28351	cytochrome P450 [*Homarus americanus*]	↑ 6.40
NP_648180	CG13675-PA [*Drosophila melanogaster*]	↑ 6.31
AAS93718	CG30022 [*Drosophila melanogaster* (fruit fly)]	↑ 6.17
BAD32189	Nup160 nucleoporin 160 [*Mus musculus* (house mouse)]	↑ 6.13
XP_455853	hypothetical protein [*Kluyveromyces lactis*]	↑ 5.93
NP_503838	G-protein coupled receptor. [*Caenorhabditis elegans*]	↑ 5.77
CAJ19121	vitellogenin receptor [*Blattella germanica*]	↑ 5.54
ZP_01117305	Zebrafish DNA sequence from clone CH211-69O18 in linkage group 16, complete sequence	↑ 5.53

XP_394275	nucleolar GTP-binding 1-like protein [*Apis mellifera* (honey bee)]	↑ 5.35
EAA08205	AGAP002490-PA [*Anopheles gambiae*]	↑ 5.28
CAG01996	unnamed protein product [*Tetraodon nigroviridis*]	↑ 5.23
AAH18737	brain protein I3 [*Homo sapiens* (human)]	↑ 5.09
AAH62838	hydroxysteroid dehydrogenase like 2 [*Danio rerio* (zebrafish)]	↑ 5.03
CAG01937	unnamed protein product [*Tetraodon nigroviridis*]	↑ 5.02
XP_973337	homocysteine-responsive endoplasmic reticulum-resident ubiquitin-like domain member 2 protein [*Tribolium castaneum* (red flour beetle)]	↑ 4.85
EAA05974	ribosomal protein S3a [*Tribolium castaneum* (red flour beetle)]	↑ 4.72
CAG05006	unnamed protein product [*Tetraodon nigroviridis*]	↑ 4.70
XP_966633	histone deacetylase Rpd3 *[Tribolium castaneum* (red flour beetle)].	↑ 4.54
CAJ02875	hypothetical protein [*Leishmania major*]	↑ 4.40
AAM09808	Sec31p [*Yarrowia lipolytica*]	↑ 4.10
ZP_01231398	hypothetical protein CdifQ_02001672 [*Clostridium difficile*]	↑ 4.06
XP_360078	predicted protein [*Magnaporthe grisea*]	↑ 3.97
XP_953782	TA16735 hypothetical protein [*Theileria annulata*]	↑ 3.89
XP_827581	hypothetical protein [*Trypanosoma brucei brucei*]	↑ 3.82
ABA25160	transposase [*Anabaena variabilis*]	↑ 3.47
YP_511438	FAD dependent oxidoreductase [*Jannaschia* sp.]	↑ 3.47
EAR85282	phospholipid-translocating P-type ATPase, flippase family protein [*Tetrahymena thermophila*]	↑ 3.35
XP_973543	polypeptide N-acetylgalactosaminyltransferase 5 [*Tribolium castaneum* (red flour beetle)]	↑ 3.21
XP_623167	PREDICTED: similar to ENSANGP00000026584 [*Apis mellifera*]	↑ 3.16
XP_972276	dolichyl pyrophosphate Man9GlcNAc2 alpha-1,3-glucosyltransferase [*Tribolium castaneum* (red flour beetle)]	↑ 3.15
EAT43025	AAEL005513-PA [*Aedes aegypti* (yellow fever mosquito)]	↑ 2.97
CAF23258	hypothetical protein [*Candidatus Protochlamydia amoebophila*]	↑ 2.90
AAX55746	low mass masquerade-like protein [*Pacifastacus leniusculus*]	↑ 2.62
EAA04645	AGAP007365-PA [*Anopheles gambiae*]	↑ 2.61
XP_855127	PREDICTED: hypothetical protein XP_850034 [*Canis familiaris*]	↑ 2.51
YP_547797	hypothetical protein [*Polaromonas* sp.]	↑ 2.26
AAM12270	GH12942p [*Drosophila melanogaster*]	↑ 1.93
EAL28826	Dpse\GA19957 [*Drosophila pseudoobscura pseudoobscura*]	↑ 1.85
XP_393169	beta-catenin-like protein 1-like [*Apis mellifera* (honey bee)]	↑ 1.76

Gene ID	**Gene description [species]**	**Clean**
AAH77956	dymeclin [*Xenopus laevis* (African clawed frog)]	↓ -1.97
XP_001071588	PREDICTED: similar to keratin associated protein 10-10 isoform 1 [*Rattus norvegicus*]	↓ -2.04
XP_688421	PREDICTED: similar to peroxisomal biogenesis factor 14 [*Danio rerio*]	↓ -2.24
AAZ75599	CRISP-ENH2 [*Enhydris polylepis*]	↓ -2.41
AAK52091	Cth cystathionine gamma-lyase [*Rattus norvegicus* (Norway rat)]	↓ -2.48
XP_813347	UDP-glucoronosyl and UDP-glucosyl transferase [*Trypanosoma cruzi*]	↓ -2.53
XP_644052	thioredoxin peroxidase [*Dictyostelium discoideum AX4*]	↓ -2.57
EAA04524	AGAP007120-PA [*Anopheles gambiae str. PEST*]	↓ -2.60
NP_200066	family/subfamily: Dentin sialophosphoprotein-related protein [*Arabidopsis thaliana*]	↓ -2.67
XP_640534	GMP synthetase [*Dictyostelium discoideum*]	↓ -2.91
CAE73165	Hypothetical protein CBG20561 [*Caenorhabditis briggsae*]	↓ -3.05
XP_799186	similar to Angiotensin-converting enzyme, testis-specific isoform precursor (ACE-T) (Dipeptidyl carboxypeptidase I) (Kininase II) [*Strongylocentrotus purpuratus* (purple sea urchin)]	↓ -3.16
XP_746198	hypothetical protein [*Plasmodium chabaudi chabaudi*]	↓ -3.17
CAC95124	TIR/NBS/LRR protein [*Populus deltoides*]	↓ -3.37
Q8WPJ2	FSU_1166 pseudo [*Fibrobacter succinogenes*]	↓ -3.41
ABD33303	hypothetical protein MtrDRAFT_AC158502g12v1 [*Medicago truncatula*]	↓ -3.76
AAL05973	peroxinectin [*Penaeus monodon*]	↓ -3.88
EAS35804	CIMG_01158 hypothetical protein [*Coccidioides immitis RS*]	↓ -4.36
XP_971851	PREDICTED: similar to NADH-ubiquinone oxidoreductase 42 kDa subunit, mitochondrial precursor (Complex I-42KD) (CI-42KD) [*Tribolium castaneum*]	↓ -4.53
AAU18227	hypothetical protein [*Bacillus cereus*]	↓ -4.92

ABG63070	50S ribosomal protein L2 [*Chelativorans* sp.]	↓ -4.50
XP_765680	hypothetical protein TP01_0153 [*Theileria parva*]	↓ -6.88
XP_785823	PREDICTED: similar to dispatched homolog 1 [*Strongylocentrotus purpuratus*]	↓ -8.52
BAB95816	truncated hypothetical protein, similar to integrase [*Staphylococcus aureus*]	↓ -11.03
EAT48380	AAEL000585-PA [*Aedes aegypti* (yellow fever mosquito)]	↓ -15.29
XP_392710	vam6/Vps39-like protein-like [*Apis mellifera* (honey bee)]	↓ -21.04
AAT61403	prolyl oligopeptidase [*Bacillus thuringiensis*]	↓ -2187.35
CAG04404	unnamed protein product [*Tetraodon nigroviridis*]	↓ -5409.11
AAZ45366	Daro_0609 peptidase M23B [*Dechloromonas aromatica*]	↓ -9110.36
NP_922637	Os10g0550300 [*Oryza sativa Japonica Group*]	↓ -10936.32
AAH90827	protease, serine, 60.2 [*Danio rerio* (zebrafish)]	↓ -15512.09
XP_465194	putative speckle-type POZ protein [*Oryza sativa* (japonica cultivar-group)]	↓ -16218.71
CAJ28140	CD9 antigen [*Dasyatis akajei*]	↓ -18236.02
NP_014128	non-motor actin binding protein [*Saccharomyces cerevisiae*]	↓ -21296.16
BAE56654	unnamed protein product [*Aspergillus oryzae*]	↓ -24885.83
AAH74846	transmembrane protease, serine 3 [*Homo sapiens* (human)]	↓ -25489.30
AAY54998	IP06749p [*Drosophila melanogaster*]	↓ -27711.51
P36308	Outer capsid protein VP4 (Hemagglutinin) [*Caenorhabditis elegans*]	↓ -28918.30
XP_624252	histone-lysine N-methyltransferase trr [*Apis mellifera* (honey bee)]	↓ -31266.20
CAG76660	ECA_RS18580 hypothetical protein [*Pectobacterium atrosepticum*]	↓ -33727.01
AAS53787	AGOS_AFR416C AFR416Cp [*Ashbya gossypii*]	↓ -37790.57
CAD51508	PFE0715w asparagine-tRNA ligase, putative [*Plasmodium falciparum 3D7*]	↓ -39926.89
ZP_00637578	hypothetical protein SfriDRAFT_3544 [*Shewanella frigidimarina*]	↓ -45161.62
AAM74161	Pax-6 protein [*Euprymna scolopes*]	↓ -46398.67
AAW88416	Serpentine receptor, class bc (class b-like) protein 36 [*Caenorhabditis elegans*]	↓ -53891.71
ABG52939	Tery_3906 hypothetical protein [*Trichodesmium erythraeum IMS101*]	↓ -60067.61
EAL26781	Dpse\GA20714 [*Drosophila pseudoobscura pseudoobscura*]	↓ -66170.96
EAR91277	transmembrane protein, putative [*Tetrahymena thermophila SB210*]	↓ -74204.27
NP_008814	NADH dehydrogenase subunit 6 [*Mustelus manazo* (starspotted smooth-hound)]	↓ -79749.44
XP_736601	hypothetical protein [*Plasmodium chabaudi chabaudi*]	↓ -82206.53
AAZ69264	MBAR_RS01565 hypothetical protein [*Methanosarcina barkeri*]	↓ -85847.78
ABG52453	Tery_3351 peptidase M23B [*Trichodesmium erythraeum*]	↓ -87307.18
XP_665805	hypothetical protein [*Cryptosporidium hominis*]	↓ -88792.28
AAH88813	lectin, galactoside-binding, soluble, 9B [*Xenopus (Silurana) tropicalis* (western clawed frog)]	↓ -114447.56
AAT64428	pMGA 1.4 [*Mycoplasma gallisepticum*]| haemagglutinin homologue	↓ -114453.63
EAT40951	AAEL007376-PA [*Aedes aegypti* (yellow fever mosquito)]	↓ -119236.95
YP_476405	CYB_0141 hypothetical protein [Synechococcus sp. JA-2-3B'a(2-13)]	↓ -129500.50
CAA17132	putative protein [*Arabidopsis thaliana*]	↓ -137110.85
CAD67790	double stranded RNA-activated protein kinase 1 [*Tetraodon nigroviridis*]	↓ -147548.17
ZP_00370063	conserved hypothetical protein [*Campylobacter upsaliensis*]	↓ -151124.91
XP_827078	hypothetical protein [*Trypanosoma brucei brucei*]	↓ -171478.98
BAD94515	peroxisome proliferator-activated receptor gamma [*Oncorhynchus keta*]	↓ -194969.42

b) F0 vs. F12 carbendazim

Symbol	Gene description [species]	CBZ (fold change)
XP_700169	PREDICTED: similar to conserved hypothetical protein [*Danio rerio*]	↑ 83178.68
ZP_01011697	outer membrane lipoprotein Blc [*Rhodobacterales bacterium*]	↑ 75647.24
XP_500810	SEC16, ISOFORM F; hypothetical protein [*Yarrowia lipolytica*]	↑ 65357.29
AAM51523	Hypothetical protein C28G1.6 [*Caenorhabditis elegans*]	↑ 38447.13
XP_765680	hypothetical protein TP01_0153 [*Theileria parva*]	↑ 16642.07
AAQ75727	NADH dehydrogenase I [*Errhomus variabilis*]	↑ 13302.91
XP_784306	PREDICTED: similar to placental protein 11 related [*Strongylocentrotus purpuratus*]	↑ 6822.25
AAN73266	polyprotein [Tick-borne encephalitis virus]	↑ 5696.41
AAH81106	MGC83377 protein [*Xenopus laevis*]; solute carrier family 5 (sodium/glucose cotransporter), member 2	↑14.93
CAD43195	disrupted in schizophrenia 1 protein [*Danio rerio*]	↑ 10.50

EAL25204	GA13016-PA [*Drosophila pseudoobscura*]	↑ 9.75
XP_829305	tyrosine phosphatase [*Trypanosoma brucei*]	↑ 8.79
AAK41879	Conserved hypothetical protein [*Sulfolobus solfataricus*]	↑ 8.10
ZP_00120785	COG2378: Predicted transcriptional regulator [*Bifidobacterium longum*]	↑ 7.77
XP_874527	PREDICTED: similar to keratin associated protein 10-7 [*Bos taurus*]	↑ 7.52
EAT35209	4-aminobutyrate aminotransferase [*Aedes aegypti*]	↑ 7.41
EAL28674	GA15521-PA [*Drosophila pseudoobscura*]	↑ 7.30
XP_971017	PREDICTED: zinc transporter ZIP11 isoform X2 [*Tribolium castaneum*]	↑ 7.15
XP_973490	PREDICTED: similar to CG2918-PA [*Tribolium castaneum*]; hypoxia up-regulated protein 1	↑ 7.07
NP_039078	Ankyrin repeat gene family protein [*Fowlpox virus*]	↑ 6.68
EAA10981	ENSANGP00000011510 [*Anopheles gambiae*]	↑ 6.51
CAA73128	Irp3 protein [*Yersinia enterocolitica*]	↑ 6.43
XP_974201	39S ribosomal protein L44, mitochondrial	↑ 6.20
AAV34884	ribosomal protein S27 [*Bombyx mori*]	↑ 6.12
XP_500452	hypothetical protein [*Yarrowia lipolytica*]	↑ 6.08
XP_421566	PREDICTED: similar to MAWD binding protein (Unknown protein 32 from 2D-page of liver tissue) [*Gallus gallus*]	↑ 5.74
EAT34816	glycine cleavage system h protein [*Aedes aegypti*]	↑ 5.60
AAC27659	tryptophan oxygenase [*Anopheles gambiae*]	↑ 5.22
XP_975769	cytochrome b-c1 complex subunit 2, mitochondrial [*Tribolium castaneum*]	↑ 5.13
ABF94587	Cystatin, putative [*Oryza sativa* (japonica cultivar-group)]	↑ 4.92
XP_974675	zinc transporter ZIP1 [*Tribolium castaneum*]	↑ 4.82
XP_361380	hypothetical protein MG03854.4 [*Magnaporthe grisea* 70-15]	↑ 4.74
AAT74669	cysteine-rich secreted protein 3 [*Mesocestoides vogae*]	↑ 4.64
XP_969056	ubiquitin carboxyl-terminal hydrolase 14 [*Tribolium castaneum* (red flour beetle)]	↑ 4.73
XP_966449	PREDICTED: similar to CG10092-PA [*Tribolium castaneum*]	↑ 4.72
EAT36570	alcohol dehydrogenase [*Aedes aegypti*]	↑ 4.72
EAR99683	Pre-mRNA cleavage complex II protein Clp1, putative; hypothetical protein TTHERM_00590200 [*Tetrahymena thermophila* SB210]	↑ 4.71
XP_001102209	PREDICTED: similar to Methylmalonyl-CoA epimerase, mitochondrial precursor (DL-methylmalonyl-CoA racemase) isoform 1 [*Macaca mulatta*]	↑ 4.68
AAZ14281	proteophosphoglycan 5 [*Leishmania* major strain Friedlin]	↑ 4.67
XP_809835	subtilisin-like serine peptidase [*Trypanosoma cruzi* strain CL Brener]	↑ 4.62
ABA02335	NADH dehydrogenase subunit 2 [*Daphnia magna*]	↑ 4.59
XP_797537	PREDICTED: similar to membrane protein mKirre [*Strongylocentrotus purpuratus*]	↑ 4.57
XP_967940	PREDICTED: similar to sideroflexin 2 [*Tribolium castaneum*]	↑ 4.57
ABF51294	NADPH-specific isocitrate dehydrogenase	↑ 4.51
YP_473323	ORF135 peptide [Hyphantria cunea nucleopolyhedrovirus]	↑ 4.51
EAS03884	Viral A-type inclusion protein repeat containing protein [*Tetrahymena thermophila*]	↑ 4.43
CAG31427	annexin A11	↑ 4.39
AAH90678	hypothetical protein [*Danio rerio*]	↑ 4.36
XP_646129	putative Nek family protein kinase [*Dictyostelium discoideum*]	↑ 4.31
XP_393344	PREDICTED: similar to hypothetical protein 18 [*Apis mellifera*]	↑ 4.27
CAC44629	deafness dystonia protein [*Takifugu rubripes*]	↑ 4.18
AAH95821	Snx9l protein; monooxygenase, DBH-like 1, like [*Danio rerio* (zebrafish)]	↑ 4.17
EAS36540	DNA polymerase epsilon catalytic subunit A [*Coccidioides immitis*]	↑ 4.10
AAR37644	molybdopterin-guanine dinucleotide biosynthesis protein [uncultured bacterium 439]	↑ 4.07
AAR04057	sulfatase FP1b [*Danio rerio*]	↑ 4.05
AAX24666	SJCHGC03634 protein [*Schistosoma japonicum*]	↑ 4.04
XP_392990	PREDICTED: similar to CG8385-PB, isoform B [*Apis mellifera*]	↑ 4.04
XP_397355	transmembrane protein 64-like [*Apis mellifera*]	↑ 3.99
AAS94231	legumain-like protease precursor [*Ixodes ricinus*]	↑ 3.97
BAC98829	F-box WD40 protein [*Labidochromis caeruleus*]	↑ 3.89
XP_791484	pREDICTED: similar to beta-1,4-mannosyltransferase, partial [*Strongylocentrotus purpuratus*]	↑ 3.84
XP_966285	DNA polymerase epsilon catalytic subunit A [*Plasmodium falciparum*]	↑ 3.83
BAB74771	alr3072 [*Nostoc* sp. PCC 7120]	↑ 3.81
XP_967132	PREDICTED: similar to CG6016-PB, isoform B isoform 1 [*Tribolium castaneum*]	↑ 3.81
XP_502986	hypothetical protein [*Yarrowia lipolytica*]	↑ 3.79
EAT45353	conserved hypothetical protein [*Aedes aegypti*]	↑ 3.78
ABA94886	Leucine Rich Repeat family protein, expressed [*Oryza sativa* (japonica cultivar-	↑ 3.76

	group)]	
XP_001075301	PREDICTED: similar to CG13731-PA [Rattus norvegicus]	↑ 3.75
AAH77956	dymeclin [*Xenopus laevis* (African clawed frog)]	↑ 3.71
XP_623472	PREDICTED: similar to ENSANGP00000024947 [*Apis mellifera*]	↑ 3.68
XP_975592	programmed cell death protein 6 [*Tribolium castaneum* (red flour beetle)]; PREDICTED: similar to CG40410-PA.3 [*Tribolium castaneum*]	↑ 3.67
XP_829735	hypothetical protein Tb11.01.8530 [*Trypanosoma brucei* TREU927]	↑ 3.63
XP_541754	guanyl-nucleotide exchange factor [*Canis familiaris*]	↑ 3.58
XP_393519	PREDICTED: similar to fatty acid-biding protein [*Apis mellifera*]	↑ 3.56
XP_971885	PREDICTED: similar to CG9762-PA [*Tribolium castaneum*]	↑ 3.56
EAL34060	GA18149-PA [*Drosophila pseudoobscura*]	↑ 3.50
ZP_01103786	conserved hypothetical protein [gamma proteobacterium KT 71]	↑ 3.47
XP_541851	PREDICTED: similar to Stabilin-1 precursor (FEEL-1 protein) (MS-1 antigen) [*Canis familiaris*]	↑ 3.45
XP_393525	PREDICTED: similar to GA20008-PA [*Apis mellifera*]	↑ 3.44
AAH24612	basic transcription factor 3-like 4 [*Mus musculus* (house mouse)]	↑ 3.43
EAT40746	conserved hypothetical protein [*Aedes aegypti*]	↑ 3.37
XP_623417	PREDICTED: similar to ENSANGP00000021084 [*Apis mellifera*]	↑ 3.35
AAK25797	delta-9 desaturase 3 [*Acheta domesticus*]	↑ 3.31
CAJ17256	ribosomal protein L10Ae [*Biphyllus lunatus*]	↑ 3.25
AAH42230	Ribosomal protein S1a protein [*Xenopus laevis*]	↑ 3.23
ZP_01376161	hypothetical protein Ccur5_01001129 [*Campylobacter curvus* 525.92]	↑ 3.16
XP_001076360	PREDICTED: similar to retinoblastoma binding protein 6 isoform 1 isoform 2 [*Rattus norvegicus*]	↑ 3.16
XP_971417	ATP synthase mitochondrial F1 complex assembly factor 1 [*Tribolium castaneum* (red flour beetle)]	↑ 3.10
CAG00408	unnamed protein product [*Tetraodon nigroviridis*]	↑ 3.05
ZP_01029146	hypothetical protein Badol_01000893 [*Bifidobacterium adolescentis*]	↑ 2.99
XP_691505	PREDICTED: similar to obscurin, cytoskeletal calmodulin and titin-interacting RhoGEF [*Danio rerio*]	↑ 2.98
CAE68115	Hypothetical protein CBG13758 [*Caenorhabditis briggsae*]	↑ 2.85
EAA05425	ENSANGP00000012700 [*Anopheles gambiae* str. PEST]	↑ 2.81
EAT44525	Niemann-Pick Type C-2, putative [*Aedes aegypti*]	↑ 2.79
AAS55566	MST84DC [*Drosophila simulans*]	↑ 2.78
NP_079004	succinyl-CoA:glutarate-CoA transferase [*Homo sapiens* (human)]	↑ 2.66
CAI86921	conserved protein of unknown function ; putative membrane protein [*Pseudoalteromonas haloplanktis* TAC125]	↑ 2.65
AAH44037	Cg8286-prov protein [*Xenopus laevis*]	↑ 2.65
YP_258141	hypothetical protein PFL_1010 [*Pseudomonas fluorescens* Pf-5]	↑ 2.62
XP_549134	PREDICTED: similar to sushi-repeat-containing protein, X-linked 2 [*Canis familiaris*]	↑ 2.58
AAQ23388	Rab7 [*Aiptasia pulchella*]	↑ 2.56
EAA12881	AGAP007952-PA [*Anopheles gambiae str. PEST*]	↑ 2.55
BAB05837	BH2118 [*Bacillus halodurans* C-125]	↑ 2.53
XP_636901	RING zinc finger-containing protein [*Dictyostelium discoideum AX4*]	↑ 2.53
ZP_00131989	COG3106: Predicted ATPase [*Haemophilus somnus* 2336]	↑ 2.44
EAT48619	serine/threonine-protein kinase vrk [*Aedes aegypti*]	↑ 2.40
CAC95124	TIR/NBS/LRR protein [*Populus deltoides*]	↑ 2.38
XP_699475	REDICTED: similar to Sympk protein [*Danio rerio*]	↑ 2.31
XP_980893	PREDICTED: hypothetical protein LOC442837 [*Mus musculus*]	↑ 2.27
XP_511735	PREDICTED: similar to hypothetical protein FLJ22175 [*Pan troglodytes*]	↑ 2.19
EAA00702	AGAP011988-PA *[Anopheles gambiae* str. PEST]	↑ 2.15
XP_625439	insulinase like peptidase [*Cryptosporidium parvum* Iowa II]	↑ 2.12
AAD50987	prothymosin a14 [*Homo sapiens*]	↑ 2.10
XP_973265	ribosome biogenesis protein BOP1 homolog [*Tribolium castaneum* (red flour beetle)]	↑ 2.08
EAA13751	AGAP010769-PA	↑ 1.94
NP_649657	Zinc-finger protein [*Drosophila melanogaster* (fruit fly)]	↑ 1.75
EAS00551	hypothetical protein TTHERM_00409040 [*Tetrahymena thermophila* SB210]	↑ 1.73
XP_710938	Potential fungal zinc cluster transcription factor [*Candida albicans*]	↑ 1.73
XP_686827	PREDICTED: similar to Bmp1 protein [*Danio rerio*]	↑ 1.56

Symbol	Gene description [species]	CBZ
NP_872312	melanoma antigen family B10 [*Homo sapiens* (human)]	↓ -1.57
XP_971465	PREDICTED: similar to Tubulin tyrosine ligase-like protein 2 (Testis-specific protein NYD-TSPG) [*Tribolium castaneum*]	↓ -1.63
AAN36896	conserved Plasmodium protein, unknown function [*Plasmodium falciparum 3D7*]	↓ -1.71
BAE73006	hypothetical protein [*Macaca fascicularis*]	↓ -1.71
EAT47168	serine protease [*Aedes aegypti*]	↓ -1.89
NP_001026467	serine/arginine-rich splicing factor 11 [*Gallus gallus* (chicken)]	↓ -1.89
NP_523475	Salivary gland secretion 1 CG3047-PA [*Drosophila melanogaster*]	↓ -1.96
XP_678020	Triops cancriformis mitochondrial DNA, complete genome [*Plasmodium berghei*]	↓ -2.04
EAR83186	WD domain, G-beta repeat protein [*Tetrahymena thermophila SB210*]	↓ -2.20
CAE61304	Hypothetical protein CBG05137 [*Caenorhabditis briggsae*]	↓ -2.23
XP_991669	PREDICTED: similar to pericardin CG5700-PB [*Mus musculus*]	↓ -2.70
AAO12215	trypsin [*Aplysina fistularis*]	↓ -3.13
ZP_01223527	4-carboxy-2-hydroxymuconate-6-semialdehyde dehydrogenase [marine gamma proteobacterium HTCC2207]	↓ -3.16
EAS02587	AMP-binding enzyme family protein [*Tetrahymena thermophila* SB210]	↓ -3.43
BAD63461	phage-related protein [*Bacillus clausii* KSM-K16]	↓ -3.48
CAA20431	GTPase regulator (predicted) [*Schizosaccharomyces pombe 972h-*]	↓ -3.70
ZP_01034049	His/Glu/Gln/Arg/opine family ABC transporter, permease protein [*Roseovarius* sp. 217]	↓ -3.71
ZP_01133737	lipase, putative [*Pseudoalteromonas tunicata* D2]	↓ -3.80
AAH85561	Hypothetical protein [*Danio rerio*]	↓ -3.84
XP_969486	PREDICTED: similar to Jagged-1 precursor (Jagged1) [*Tribolium castaneum*]	↓ -4.03
NP_611397	CG15111-PA, isoform A [*Drosophila melanogaster*]	↓ -4.09
XP_971073	AP-1 complex subunit sigma-2 [*Tribolium castaneum* (red flour beetle)]	↓ -4.12
XP_970811	vesicle transport protein SEC20 [Tribolium castaneum (red flour beetle)]	↓ -4.17
XP_624856	BTB/POZ domain-containing protein KCTD5-like [*Apis mellifera* (honey bee)]	↓ -4.18
XP_380554	Hypothetical protein FG00378.1 [*Gibberella zeae* PH-1]	↓ -4.21
XP_392015	PREDICTED: similar to CG31116-PC, isoform C [*Apis mellifera*]	↓ -4.22
XP_426719	PREDICTED: similar to seven in absentia homolog 2; seven in absentia [*Drosophila*] homolog 2 [*Gallus gallus*]	↓ -4.46
ZP_01304133	xylosidase/arabinosidase [*Sphingomonas* sp. SKA58]	↓ -4.54
XP_523482	PREDICTED: hypothetical protein [*Pan troglodytes*]	↓ -4.56
AAD55141	di-domain hemoglobin precursor [*Daphnia pulex*]	↓ -4.61
XP_623750	myotrophin-like [*Apis mellifera* (honey bee)]	↓ -4.85
XP_397115	PREDICTED: similar to ENSANGP00000014264 [*Apis mellifera*]	↓ -4.98
EAL17999	hypothetical protein [*Cryptococcus neoformans var. neoformans B-3501A*]	↓ -4.98
BAE38837	Zranb2 zinc finger, RAN-binding domain containing 2 [*Mus musculus* (house mouse)]	↓ -5.08
XP_995118	PREDICTED: hypothetical protein [*Mus musculus*]	↓ -5.14
AAO27090	CTP synthase [*Buchnera aphidicola* str. Bp (Baizongia pistaciae)]	↓ -5.20
EAA09458	AGAP005124-PA [*Anopheles gambiae str. PEST*]	↓ -5.25
XP_394551	NEDD8-conjugating enzyme UBE2F-like [*Apis mellifera* (honey bee)]	↓ -5.26
XP_549046	PREDICTED: similar to hephaestin isoform a [*Canis familiaris*]	↓ -5.26
EAT46404	conserved hypothetical protein [*Aedes aegypti*]	↓ -5.36
XP_966534	ethanolamine-phosphate cytidylyltransferase [*Tribolium castaneum* (red flour beetle)]	↓ -5.37
EAT43058	AAEL005474-PA [*Aedes aegypti* (yellow fever mosquito)]	↓ -5.40
NP_650751	CG7718 [*Drosophila melanogaster*]	↓ -5.42
AAS48991	UL112/113 [*Human herpesvirus* 5]	↓ -5.47
BAB21109	Ef-1d elongation factor 1 delta [*Bombyx mori* (domestic silkworm)]	↓ -5.51
EAL26005	Dpse\GA14100 [*Drosophila pseudoobscura pseudoobscura*]	↓-5.60
EAT38914	AAEL009235-PA [*Aedes aegypti* (yellow fever mosquito)]	↓ -5.64
CAA84321	Hypothetical protein F45H7.1 [*Caenorhabditis elegans*]	↓ -5.66
XP_455853	unnamed protein product [*Kluyveromyces lactis*]	↓ -5.88
ABB06938	FAD dependent oxidoreductase [*Burkholderia* sp. 383]	↓ -6.10
CAH90002	EIF4A2 eukaryotic translation initiation factor 4A2 [*Pongo abelii* (Sumatran orangutan)]	↓ -6.21
CAK11279	novel protein similar to vertebrate topoisomerase (DNA) II beta 180kDa (TOP2B) [*Danio rerio*]	↓ -6.54
XP_682935	Uncharacterized protein. Subfamily: SOLUTE CARRIER FAMILY 35 MEMBER F1 (PTHR14233:SF10) [*Danio rerio*]	↓ -6.55

XP_796182	PREDICTED: similar to SHQ1 homolog [*Strongylocentrotus purpuratus*]	↓ -6.22
ABF51368	H^+ transporting ATP synthase O subunit [*Bombyx mori* (domestic silkworm)]	↓ -6.64
XP_785816	PREDICTED: similar to muscle Y-box protein YB2 [*Strongylocentrotus purpuratus*]	↓ -6.66
EAS03921	HMG box protein [*Tetrahymena thermophila SB210*]	↓ -6.68
XP_967013	PREDICTED: similar to CG9160-PA, isoform A [*Tribolium castaneum*]	↓ -6.77
ZP_01065094	hypothetical protein MED222_15549 [*Vibrio* sp. MED222]	↓ -6.84
XP_001072503	PREDICTED: similar to putative MAPK activating protein PM20,PM21 isoform 1 [*Rattus norvegicus*]	↓ -6.92
CAG09120	unnamed protein product [*Tetraodon nigroviridis*]	↓ -7.05
YP_258814	cytochrome c-type biogenesis protein CycH [*Pseudomonas fluorescens* Pf-5]	↓ -7.13
XP_973533	malate dehydrogenase, mitochondrial [*Tribolium castaneum* (red flour beetle)]	↓ -7.14
AAH76191	thoc7 THO complex 7 [*Danio rerio* (zebrafish)]	↓ -7.15
CAF94261	unnamed protein product [*Tetraodon nigroviridis*]	↓ -7.20
CAG05207	unnamed protein product [*Tetraodon nigroviridis*]	↓ -7.30
XP_976221	PREDICTED: similar to CG4944-PB, isoform B isoform 2 [Tribolium castaneum]	↓ -7.33
XP_968064	40S ribosomal protein S3a [*Tribolium castaneum* (red flour beetle)]	↓ -7.36
NP_077791	lipoic acid synthetase [Mus musculus]	↓ -7.49
AAV31414	26S protease regulatory subunit-like protein [*Toxoptera citricida*]	↓ -7.65
AAX28551	SJCHGC05463 protein [*Schistosoma japonicum*]	↓ -7.66
EAA08286	ENSANGP00000017110 [*Anopheles gambiae* str. PEST]	↓ -7.73
CAB60138	putative polyprotein [Wheat yellow mosaic virus]	↓ -8.05
AAN37244	conserved Plasmodium protein, unknown function [*Plasmodium falciparum 3D7*]	↓ -8.09
CAF92521	unnamed protein product [*Tetraodon nigroviridis*]	↓ -8.50
XP_974308	metaxin-2-like [*Tribolium castaneum* (red flour beetle)]	↓ -8.57
XP_624608	renin receptor-like [*Apis mellifera* (honey bee)]	↓ -8.83
YP_052887	ribosomal protein S7 [*Saprolegnia ferax*]	↓ -8.85
AAY60144	oocyte maturation factor Mos [*Anas poecilorhyncha*]	↓ -8.87
XP_623241	growth hormone-inducible transmembrane protein-like [*Apis mellifera* (honey bee)]	↓ -9.53
AAK27862	Hypothetical protein Y37E3.4 [*Caenorhabditis elegans*]	↓ -9.96
ZP_01120123	hypothetical protein RB2501_07115 [Robiginitalea biformata HTCC2501]	↓ -12.89
XP_973543	polypeptide N-acetylgalactosaminyltransferase 5	↓ -14.60
XP_855304	PREDICTED: similar to cyclin K [*Canis familiaris*]	↓ -6377.84
YP_581584	histidine kinase [*Psychrobacter cryohalolentis* K5]	↓ -17088.07
EAM56123	conserved hypothetical protein [*Solibacter usitatus* Ellin]	↓ -49456.71
XP_729076	hypothetical protein PY01338 [*Plasmodium yoelii* yoelii]	↓ -58892.73

Chapter 7

General discussion and conclusions

General discussion and conclusions

Global changes are causing several pressures in aquatic ecosystems, making species more vulnerable and threatening its richness. Considering that in the environment compounds are often in mixtures, methodologies assessing effects of mixtures are required, mostly because compounds might act synergistically. Additionally, predicting the magnitude of these pernicious effects in organisms should be accuratelly carried out to help an effective regulatory framework and prevent deleterious effects to the environment. Until recently, the impact on several generations within populations has been negligible in environmental risk assessment. Long-term exposures at low concentrations are expected and tend to be the real scenario that risk assessors and managers have to deal with.

Many pesticides are still extensively applied, including carbendazim, which has a global market as active ingredient equivalent to 12 000 tonnes and it is classified as reproductive toxicant in Europe (with effects on fertility and development) (Gray *et al*., 1990).

Considering the above mentioned, in the present work the assessment of the toxic effect of carbendazim and triclosan single and in mixture to *D. magna* and the multigenerational effects of carbendazim considering a multigenerational approach was under investigation. In the multigenerational experiment, carbendazim was tested under an environmental relevant concentration, 5 µg/L, similar to real concentrations found in surface waters (Palma *et al*., 2004).

Several highlights can be derived from the present study:

- *Carbendazim presented higher lethal and sublethal toxicity than triclosan to D. magna.*

In the present work, carbendazim presented higher lethal and sublethal toxicity when compared to triclosan in *D. magna*, while assessing the immobilisation, reproduction, feeding activity and DNA damage (Chapter 2 - Silva *et al*. 2015). Literature with toxicity data for triclosan and using *D. magna* is still scarce; for the immobilisation a 48h-LC_{50} value of 390 µg/L was found (Orvos *et al*., 2002), which is slightly lower compared to the

obtained in the present study. To our knowledge, for the feeding activity, reproduction or DNA damage no data besides the one presented here was available. Considering carbendazim, for the immobilisation data, 48h-LC_{50} values in the literature varied from 110 to 350 µg/L (Ferreira *et al*., 2008; U.S.EPA, 2000), while for the feeding activity the EC_{50} values in literature were slighltly lower (Ferreira *et al*., 2008; Ribeiro *et al*., 2011); for the reproduction endpoint (number of neonates and aborted eggs), the EC_{50} values in literature were in the same order compared with the values determined in the present work (Ribeiro *et al*., 2011). Carbendazim mode of action is related with mitosis inhibition in animal and plant cells (Davidse, 1986), and in fact, the occurrence of aborted eggs in *D. magna* exposed to carbendazim was observed (Ribeiro *et al.* 2011, Chapter 2- Silva *et al*. 2015). Consequently, errors in mitosis can promote the acquisition of DNA damage (Ganem and Pellman, 2012), as will be discussed later.

- *Carbendazim and triclosan caused DNA damage in D. magna.*

Many environmental contaminants are described as agents that induce DNA damage to aquatic organisms and the success of the application of the comet assay on *D. magna* to assess these effects was already confirmed (Pellegri *et al*., 2014). In the present work, the comet assay demonstrated to be a suitable and sensitive technique to measure DNA strand breaks after exposure to carbendazim and triclosan in *D. magna* (Chapter 2).

Effects in terms of DNA damage for both compounds were already observed in different organisms (carbendazim: marine invertebrate *Donax f*aba, plant cells of *Hordeum vulgare* L. and triclosan: zebra mussel *Dreissena polymorpha,* algae *Closterium ehrenbergii* and earthworm *Eisenia fetida*) (Binelli *et al*., 2009; Ciniglia *et al*., 2005; JanakiDevi *et al*., 2013; Lin *et al*., 2012; Singh *et al*., 2008). However, to our knowledge, it was never determined for *D. magna*. Results showed that both compounds caused DNA damage to daphnids cells in the tested concentrations (Chapter 2). Carbendazim owns a high permeability across the lipid bilayer, which facilitates the entry into the cells, causing fragmentation and cell death (JanakiDevi *et al*., 2013; Jia *et al*., 2002). Additionally, DNA bases adenine and guanine, with purine ring, are structurally similar to carbendazim, therefore carbendazim may compete with purine of adenine and guanine, interfering with DNA synthesis (JanakiDevi *et al*., 2013). Reduction in DNA synthesis prevents

chromosomes replication, interfering with mitotic division (Farag *et al*., 2011). For triclosan, information related with possible mechanisms involved in DNA damage are sparse, however some authors reported that the DNA damage caused by triclosan might have been due to oxidative stress (and consequently production of ROS), causing cell damage (Lin *et al*., 2012).

- *The binary mixture of carbendazim and triclosan caused different patterns of mixture toxicity for different endpoints. For the DNA damage endpoint,a dose ratio deviation was observed, with synergism mainly caused by triclosan.*

Mixture patterns after daphnids exposure to the binary mixture of carbendazim and triclosan revealed to be additive for the feeding inhibition and reproduction data with a good fit by the reference model IA, meaning that compounds did not interfered with each other. For the immobilisation data, a dose level (with antagonism at low doses and synergism at high doses) was found, while for the DNA damage data a dose ratio dependency (with synergism mainly caused by triclosan) being observed (Chapter 2). In ecotoxicology, synergism is considered the worst case scenario, since there is an enhancement of toxicity and therefore is the pattern causing most concern.

Mixture experiments are usually labor-intensive, and consequently a high variability in the endpoint measured is likely to occur (Cedergreen *et al*., 2007). Cedergreen *et al.* (2007) observed that dose-dependent effects sometimes could not be consistently repeated, highlighting the need of repeating mixture toxicity tests. In the present study, reproducibility of the mixture patterns (carbendazim and triclosan) was found in the multigenerational experiment (Chapter 3) with daphnids kept in clean medium (F8 and F12). Additionally, the same pattern was found previously (Chapter 2): with a dose level dependency with antagonism observed at low doses of the chemical mixture for the immobilisation data and a dose ratio dependency with synergism mainly caused by triclosan for the DNA damage.

- *Multigenerational impacts of carbendazim to D. magna were mainly observed in terms of DNA damage, with an increase in DNA damage throughout generations.*

It should be considered that a prenatal stage exposure (in developing embryos) might affect organisms more than if exposure is carried out at juveniles or adult stages (Arndt, 2014; Perera and Herbstman, 2011). Unlike single generation experiments, multigenerational experiments might provide important information regarding changes in organisms/offspring sensitivity.

The present study investigated the multigenerational effect of carbendazim in daphnids, assessing the reproductive output, body length, longevity, DNA damage (determined by the comet assay), biochemical biomarkers (cholinesterases (ChE), catalase (CAT), glutathione *S*-transferase (GST) and lipid peroxidation (LPO)), energy-related parameters (lipids, carbohydrates, proteins and available and consumed energy) and changes in gene expression (*D. magna* custom microarray) (Chapter 3, 4, 5 e 6). DNA damage increased throughout the generations exposed to carbendazim (Chapter 3 and Chapter 5). This can raise two main hypothesis regarding a possible transfer from mother to offspring throughout generations or that the DNA repair mechanisms were probably being less effective (reduced or with slower repair) as a consequence of the toxic exposure (Atienzar and Jha, 2004; Collins *et al*., 1995).

- *Pre-exposure of daphnids to carbendazim caused a higher increase in DNA damage after pulse exposure with triclosan and binary mixture, however the pre-exposure appeared to have no effect on the immobilisation data and no differences in mixture patterns (immobilisation and DNA damage).*

In the multigenerational experiment, similar sensitivity was found for daphnids pre-exposed to carbendazim for F7, F8 and F12 after the pulse exposure to triclosan (similar LC_{50} values comparing with daphnids in clean medium) (Chapter 4). After exposure to carbendazim, triclosan and its binary combination, F12 daphnids previously exposed to carbendazim presented an overall higher percentage of DNA damage comparing with F12 daphnids in clean medium (Chapter 4). After the pulse exposure with the binary mixture (carbendazim and triclosan), an overall look of the results demonstrated similar mixture patterns between daphnids in clean medium and daphnids previously exposed to carbendazim (F8 and F12), for the immobilisation and DNA damage endpoint (Chapter 4).

Few studies are available using mixtures in multigenerational experiments and usually daphnids are exposed from the beginning and for successive generations to chemical mixtures (Brausch and Salice, 2011; Dietrich *et al*., 2010) and not to pulses of mixtures as in the present work (Chapter 4). Herein, a pre-exposure to carbendazim for several generations (F8 and F12) did not seem to affect the response to pulses with the mixture of carbendazim and triclosan (immobilisation and DNA damage data).

- *D. magna exposed for twelve generations to carbendazim presented a significant reduction in longevity comparing with daphnids always kept in clean medium.*

In the multigenerational experiment, despite the absence of effects using the intrinsic rate of natural increase (*r*) or length, changes in longevity/survival, DNA damage and gene expression were confirmed from F0 to F12 generation after the continuous exposure to carbendazim (comparing with daphnids kept in clean medium) (Chapter 5 and 6). In multigenerational experiments, survival/longevity has been assessed, though usually it was carried out only during 21 days (using standard reproduction tests) (Chen *et al*., 2013; Sánchez *et al*., 2004; Tanaka and Nakanishi, 2002). However, in the present study, longevity/survival was evaluated considering the entire lifespan (with some daphnids reaching more than 60 days). Nevertheless, Chen *et al.* (2013) observed a decrease in survival in *D. magna* exposed to pentachlorophenol at F2 generation (compared to F0). The authors stated that the magnification of the toxic effect was probably due to accumulation or maternal transfer of organic compounds to offsprings (Chen *et al*., 2013; Dietrich *et al*., 2010). The intrinsic rate of natural increase (*r*) can provide information at the population level (Buhl *et al*., 1993), it integrates the number of mothers, number of neonates, number of broods and time (days) to the brood release. In the present study, no differences in the *r* value were found between non-exposed and exposed daphnids to carbendazim, probably due to a compensation between survival, fecundity and maturation time (Zalizniak and Nugegoda, 2006). Herein, the *r* value was determined considering a 21 days period of exposure, similar as reported in other multigenerational experiments (Volker *et al*., 2013; Zalizniak and Nugegoda, 2006).

- *Carbendazim affected genes involved in response to stress, DNA replication/repair, neurotransmission, embryogenesis, protein biosynthesis, ATP production, lipids and carbohydrates metabolism in both F0 and F12 generation.*

Considering that, genomic instability might be promoted by DNA damage and abnormal mitosis (Ganem and Pellman, 2012) and that carbendazim, which is know to disturb mitosis, caused DNA damage in daphnids at relatively low concentrations, effects on gene expression levels were addressed. To our knowledge, effects of carbendazim on gene expression were never studied in *D. magna*. However, in other species, *e.g. Enchytraeus albidus*, carbendazim affected genes involved in DNA damage/DNA repair processes and in other important processes, namely regulation of the cell cycle, response to stress and microtubule-based movement (Novais *et al*., 2012). The *D. magna* microarray results suggest that carbendazim induced multiple responses, with changes in gene expression in genes involved in response to stress, DNA replication/repair, neurotransmission, embryogenesis, lipid metabolism, *etc*. (Chaper 6). Multiple responses were also observed by Jiang *et al.* (2015) in zebrafish larvae exposed to carbendazim, in which the expression of a high number of genes involved in oxidative stress, endocrine and immune systems and apoptosis pathway was changed.

Another important subject in transcriptomic analyses is the organism' age. David *et al.* (2011) observed a higher transcription of genes involved in the response to DNA damage in adult daphnids (7 d), which can be translated into greater protein activity, compared to neonates (< 24h). The authors reported that adult daphnids appeared to be more responsive to genotoxicants (induction of DNA repair genes) than neonates, and the hypothesis advanced by the authors suggested that neonates had a lower DNA repair capacity (David *et al*., 2011). Despite differences in organisms' age have not been considered in the present work, in our multigenerational approach, DNA damage results were supported by the gene expression analysis in the microarray, where some genes involved in DNA damage/repair were differentially transcribed in both generations. Relevant genes might therefore be used as biomarkers of exposure to toxicants in the environment in an early stage.

- *No clear pattern regarding changes in sensitivity in daphnids exposed to carbendazim throughout generations.*

Except for DNA damage and longevity, the ecotoxicological endpoints by themselves showed no clear changes in daphnids sensitivity throughout the multigenerational experiments. In Chapter 3 no clear pattern towards sensitivity for the immobilisation data (LC_{50} values) were found. The biochemical biomarkers ChE, GST and LPO showed differences between generations of daphnids in carbendazim comparing with daphnids kept in clean medium, yet no clear pattern was observed as well (Chapter 5). The importance of biochemical biomarkers in environmental studies was discussed by Jemec *et al.* (2010); the authors referred that, on the opposite of what was anticipated, biochemical biomarkers were not always more sensitive comparing with whole-organisms responses. Jemec *et al.* (2010) statements and the present research highlights the utmost importance of using multiple endpoints and multigenerational approaches in environmental risk assessment (Chapter 3, 4, 5 and 6).

- *D. magna demonstrated a low potential of recovery for carbendazim induced DNA damage.*

The ability of daphnids to recover after a pre-exposure to carbendazim was evaluated in the present research as well. A low potential of recovery seemed to have occurred for the DNA damage, however the time of daphnids in clean conditions was short (few days) (Chapter 3). Apparently, DNA damage was transmitted (increased) throughout generations and maintained after return to clean medium (for some days) and/or inefficiency in the mechanisms of DNA repair was occurring (Jha, 2008; Plaire *et al*., 2013). Opposite to our findings, a 9 day recovery in clean medium upon benzo(a)pyrene exposure enabled DNA repair in *D. magna* (Atienzar and Jha, 2004) and after one generation after cadmium exposure (Guan and Wang, 2006). In future work, it should be considered the extension of time of daphnids in clean medium, for instance, during some generations.

- *Gene expression changes throughout generations of D. magna exposed to carbendazim were not kept in time.*

The exposure of daphnids to carbendazim did not cause a stable change in pratically none of the endpoints measured, neither in gene expression from F0 to F12 generations. A lower number of differentially expressed genes was observed in the F12 comparing with the F0 exposed to carbendazim, where only five up-regulated genes and two down-regulated genes were common between F0 and F12 (Chapter 6).

Since a long time ago, it is stated that the greater the pressure the faster resistance develops (Crow, 1957), however the concentration used in this multigenerational experiment was low and probably did not cause a great pressure. Additionally and as previously stated, in this research no clear pattern towards tolerance was found, though this irregularity has also been observed in previous studies (Dietrich *et al*., 2010; Jeong *et al*., 2015). Dietrich *et al.* (2010) found that neither the single nor a mixture of pharmaceuticals affected the *D. magna* generations in a steady way. Nevertheless, further studies are required to explain the cause of this pattern of irregularity in some endpoints throughout the multigenerational experiments.

- *More insights on potential modes of action of carbendazim.*

Carbendazim showed to act on a wide spectrum of processes, namely on daphnids reproduction (with a decrease on the number of neonates and an increase in the number of aborted eggs with increasing concentrations of carbendazim). The use of this battery of subcellular endpoints provided a closer insight into the effects caused by carbendazim to *D. magna* but also on potential modes of action. Carbendazim acted on daphnid's DNA (caused DNA damage as DNA strand breaks), induced changes in daphnids antioxidant and detoxification systems (CAT and GST activities). Moreover, carbendazim altered the expression of several genes involved in DNA replication/repair, neurotransmission, ATP production, lipid metabolism, embryonic development, *etc.*, as previously described. These results are indicative of the potential of genomics approaches to gain insights into modes of action of compounds and should be considered to increase confidence within environmental risk assessment processes (EFSA, 2015).

Microarray data provides valuable information about the influence of chemicals on organisms at the molecular level, however to determine the precise mechanisms of action,

proteomics or metabolomics studies should be used (though these studies are quite expensive).

- *Link between subcellular and individual effects after multigenerational exposure is not simple.*

In the long-term exposure to carbendazim few effects at the individual level (practically only on daphnids longevity) or transposed to the populations could be depicted throughout generations (till F12). Subcellular effects are usually considered measures of initial changes in organisms in response to stress. In the present research, at the subcellular level an increase in DNA damage was observed jointly with a different transcription of genes (and number of genes) from F0 to F12 (Chapter 5 and 6), however, no straight relationship between subcellular and individual levels was possible to establish. Additionally, although the majority of the energy is used for growth, reproduction and basal metabolism, chemical exposure might induce compensatory changes in the energy budget of an organism (De Coen and Janssen, 2003). In the present study, a decrease in the number of neonates at almost all generations of daphnids exposed to carbendazim was found, probably indicating that the energy was being allocated for detoxification or other processes rather than for reproduction (Chapter 5).

Relationships between levels of organization have already been discussed and are quite challenging to perform and considered sometimes hard (Li *et al*., 2015). As well as sublethal responses to chemicals are ruled by general mechanisms, probably with numerous genes of small effects, while lethal responses are ruled by specific mechanisms, with fewer genes of great effects (Barata *et al*., 2000; Hoffmann and Parsons, 1997; Lopes *et al*., 2005).

- *Changes in sensitivity throughout generations of organisms kept in clean medium.*

Another output of the current work was the demonstration of changes in sensitivity throughout the generations even in organisms always kept in control, which represents similar conditions to the culture setup. This was illustrated for almost all endpoints (Chapter 3, 4 and 5), including variabiliy in LC_{50} values throughout generations (Chapter

3), and was already depicted at the gene transcription level (Chapter 6). Gene expression changes were observed even among daphnids always in control situation at different times (F0 and F12 generations) (Chapter 6). Changes in gene expression among control treatments has already been reported for daphnids and also earthworms (Owen *et al*., 2008; Vandegehuchte *et al*., 2010a; Vandegehuchte *et al*., 2010b). For biochemical biomarkers, different values were found for control organisms in the same experiment and same laboratory yet at different times (Jemec *et al*., 2010). Variability in daphnids gene expression might be attributed to differences in physiological processes, for instance, in reproductive cycles or in molting phases (Vandegehuchte *et al*. 2010a, 2010b). Despite the daphnids' age, that is the same in the different generations, there is a possibility that a difference of hours might influence some of these processes. Traudt *et al.* (2016) demonstrated that using the narrow 24h age window for *D. magna* neonates (following the standardized protocols for *D. magna*) and after exposure to cadmium, a difference of 10-folds in the EC_{50} values was observed between neonates with 0-4h when compared with neonates with 20-24h, being cadmium less toxic to the youngest neonates. The use of neonates from different broods could explain these variations as well (Barata *et al*. 2001). Additionally, although all procedures/conditions are intended to be constant a slight inherent variability is likely to occur, for instance in food quality (algae) and/or small variations in room temperature. Rose and collegues (2004) observed a decrease in sensitivity (referred by the authors as tolerance) in control cultures throughout generations using the cladoceran *Ceriodaphnia* cf. *dubia* after exposure to the pesticide 3,4-dichloroaniline. The authors enumerated possible explanations for this change in sensitivity, including the effect of experimental factors, for instance food (algae) or water quality during the test (Rose *et al*., 2004). Although this variability was observed, as Rose *et al*. (2004) stated, differences between the controls and the treatments, although not ideal, are still factual and should be argued. The implications of these findings should be considered in environmental risk assessment.

- *Future research and need for standardized protocols in multigenerational experiments to improve environmental risk assessment.*

In the mutigenerational experiment with carbendazim, changes in gene expression appeared and differences were observed when comparing F0 with F12 daphnids (Chapter 6). Albeit no direct investigations on epigenetic modifications were performed, there is a possibility that carbendazim is acting on the epigenome of *Daphnia*. Further studies including the evaluation of epigenetic modifications, for instance DNA methylation or histone modification, should be performed in multigenerational experiments.

Another interesting point to test in multigenerational experiments could be the use of low food quantity, as often happens in the environment. Organisms receiving food *ad libitum*, have resources (energy) for physiological defense (*e.g.* detoxification and/or reparation) and consequently they probably are less sensitive to chemicals, opposite to starved organisms (Pavlaki *et al*., 2014; Pieters *et al*., 2005; Sibly, 1999). Likewise, body size is an important factor that might influence chemical sensitivity, since smaller organisms (in earlier stages) might be more sensitive due to the increase in uptake and bioconcentration of chemicals (Reyes *et al*., 2015; Vesela and Vijverberg, 2006). Therefore, *Daphnia* body length should be measured in the beginning of each generation and considered in future researches.

Few studies have investigated the bioaccumulation potential of carbendazim and, to our knowledge, none used *Daphnia* sp.. Bioaccumulation might be an important factor when testing effects at the multigenerational level. Some studies reported that carbendazim has low potential to bioaccumulate (in fish) (Dang and Smit, 2008) though, earthworms showed to bioaccumulate this compound in soils with high concentrations of carbendazim (Burrows and Edwards, 2004).

Findings in the present study showed that long-term effects of a chemical might actually occur (multigenerational scenarios), with possible consequences to populations and consequently along the trophic chain. Therefore, there is a need to address multigenerational and transgenerational effects of chemicals by developing standardized protocols for these multigenerational experiments, with a view to fill the existing knowledge gaps in environmental risk assessment.

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